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Australian Grape and Wine Authority

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Organic acid metabolism and the control of grape berry acidity in a warming climate



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: UA1002

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Research Organisation: **The University of Adelaide**

Date: **December 2015**

Project UA1002:

Organic acid metabolism and the control of grape berry acidity in a warming climate

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Abbreviations

ALMT	Aluminium-activated Malate Transporter
ANOVA	Analysis of Variance
BCECF-AM	2',7'-Bis(2-carboxyethyl)-5(6)-carboxyFluorescein acetoxymethyl ester
DAF	Days After Flowering
DNA	Deoxyribonucleic Acid
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary DNA
gDNA	Genomic DNA
GABA	Gamma-aminobutyric Acid
GC/MS	Gas Chromatography / Mass Spectrometry
GDD	Growing Degree Days
gFW	Grams Fresh Weight
HPLC	High Performance Liquid Chromatography
HSP	Heat Shock Protein
2-KGA	2-Keto-L-gulonic Acid
2-KGR	2-Keto-L-gulonate Reductase
L-IDH	L-Idonate Dehydrogenase
MA	Malic Acid
MDH	Malate Dehydrogenase
cMDH	Cytosolic Malate Dehydrogenase
mMDH	Mitohondrial Malate Dehydrogenase
ME	Malic Enzyme
NAD	Nicotinamide Adenine Dinucleotide
NADH	NAD, reduced form
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	NADP, reduced form

NAD-ME	NAD-dependent Malic Enzyme
NADP-ME	NADP-dependent Malic Enzyme
NAD-MDH	NAD-dependent Malate Dehydrogenase
NADP-MDH	NADP-dependent Malate Dehydrogenase
NIST	National Institute of Standards and Technology
OEX	Overexpression
PCR	Polymerase Chain Reaction
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate Carboxylase
PEPCK	Phosphoenolpyruvate Carboxykinase
PK	Pyruvate Kinase
PPase	Pyrophosphatase
PPDK	Pyruvate, Pi Dikinase
RNA	Ribonucleic Acid
RNAi	RNA interference
SD	Standard Deviation
SEM	Standard Error of the Mean
TA	Tartaric Acid
TCA	Tricarboxylic Acid
TDT	Tonoplast Dicarboxylate Transporter
TSS	Total Soluble Solids

1. Abstract

The objective of this project was to identify potential targets for the manipulation of organic acid profiles in grapes, with a long-term goal of minimising the impact of climate change on grape must acidity. Transgenic grapevines were developed to better understand how acidity is regulated within berries and leaves. New metabolic models were generated from field- and chamber-based temperature experiments and from cultivars with inherently different acid profiles. These demonstrated correlative links between organic acid and amino acid metabolism. Therefore altering nitrogen supply may provide a relatively straightforward means for manipulating berry acid levels, warranting further investigation.

2. Executive summary

Two strategies could be used to combat low-acidity in grapes grown during hot seasons. The first is to identify a management tool to reduce the loss of malic acid upon exposure of the vine to elevated temperatures. The second is to increase levels of tartaric acid in the fruit, such that losses of acidity due to malic acid degradation are compensated by an abundance of heat-stable tartaric acid. This project aimed to advance progress on both of these strategies and could thus be divided into two general aims. The first was to pinpoint important regulatory junctions of malic acid metabolism that may be targeted for reducing acid losses during hot periods of the season. The second was to discover new genes involved in the largely uncharacterised tartaric acid biosynthesis pathway, such that tartaric acid production may be manipulated to control acidity in the berry regardless of seasonal temperature.

To address the first aim, elevated temperature treatments in field and controlled-environment (chamber) conditions were used to explore the effects on various genes involved in malic acid metabolism, as well as the effects on other metabolite pools within the berry.

Based on gene transcript levels, three distinct areas of malic acid metabolism seem to be affected by elevated temperature: some malic acid synthesis enzymes were down-regulated, some malic acid degradation enzymes were up-regulated, and some malic acid transporters were affected. Overall, improving the ability of a cell to compartmentalise malic acid such that it is protected from degradation, as well as improving the ratio of enzyme activities for malic acid synthesis relative to degradation, would decrease the likelihood that malic acid will encounter an enzyme capable of degrading it, and thus could help to retain higher levels of malic acid in response to elevated temperatures or during extended ripening periods.

Based on metabolite levels, there was a negative correlation between malic acid and amino acid levels, which suggested that a change in the balance of carbon and nitrogen pools in the fruit could alter malic acid metabolism. This was consistent with some of the observed shifts in the expression of malic acid-metabolising enzymes, which can act as branch-points between organic acid and amino acid metabolism. It was also consistent with data from a grapevine cultivar comparison conducted within this study. This suggests that the levels of organic acids in the fruit at harvest may be malleable, through the management of nitrogen levels. In the literature there are some references to altered acidity of berries when nitrogen status is altered, but this has not been closely investigated as a management tool for malic acid levels. The effect of nitrogen supply on the transcript levels and activities of genes involved in malic acid metabolism seems to be an important way forward in this

area.

To address the second aim, candidate genes for uncharacterised steps of the tartaric acid biosynthesis pathway were explored to determine whether the corresponding enzymes could in fact carry out the expected reactions.

For one of these genes, the expected activity could indeed be demonstrated, however the enzyme was far more active with some alternative substrates, and therefore the primary role of this gene product was unlikely to be tartaric acid biosynthesis. However, this may be an example of a biosynthetic pathway commandeering enzymes from other pathways and performing multiple functions within the cell. In addition, the already-known enzyme of the tartaric acid biosynthesis pathway in grapevine was recently shown to have diverged from an enzyme family involved in sorbitol metabolism in the *V. vinifera* genome. Therefore, when specific tartaric acid precursors are available in the grape berry cell, this new candidate enzyme could be capable of utilising them for tartaric acid biosynthesis. However, when the precursors are not available this enzyme may carry out other functions, as may be the case for more than one step in the tartaric acid biosynthetic pathway. In order to confirm whether this candidate enzyme contributes to tartaric acid biosynthesis, it is necessary to determine whether tartaric acid levels are affected by altering expression of this gene in grapevine. Transgenic plants for such an investigation have been generated during this project and await further analysis.

Transgenic grapevines were also generated to target other genes thought to be involved in organic acid metabolism, in a regulatory (i.e. rate-limiting) capacity. A number of plants were generated, targeting different genes of the tartaric acid biosynthesis pathway, including the newly characterised gene mentioned above. These plants still need to be analysed comprehensively in order to determine the effects on metabolite levels in the leaves and/or fruit.

Acknowledgements

Research and activities of the project covered by this report were supported financially by Australia's grape growers and wine makers through their investment body the Grape and Wine Research and Development Corporation (GWRDC), with matching funds from the Australian Government.

Additional funding to support pilot trials using growth chamber experiments at The Plant Accelerator was supplied by Wine2030 Research Network (The University of Adelaide Wine Future, UAWF)

Field treatments were carried out at the Coombe vineyard, Urrbrae, South Australia and at the Nuriootpa Research Station, South Australia in collaboration with Prof. Victor Sadras at the South Australian Research and Development Institute (SARDI).

Metabolomic work was carried out in collaboration with the Australian Wine Research Institute (AWRI), Urrbrae, South Australia and Dr Robert Hancock at The James Hutton Institute, Dundee, Scotland.

All microvine work was carried out in collaboration with Dr Mark Thomas at CSIRO Plant Industry, Urrbrae, South Australia.

Fruit of *Ampelopsis aconitifolia* vine were kindly donated by The Adelaide Botanic Gardens, Adelaide, South Australia.

3. Background

The concentration of organic acids is the most determining factor for grape berry acidity at harvest. Acidity, which is pivotal in the use of grapes for winemaking, confers desirable organoleptic properties and aging capacity on wines. Acidity moreover enables juice to withstand oxidation and spoilage from chemical and microbial sources. The two major organic acids of grape berries, malic acid and tartaric acid are synthesised through different metabolic pathways, and are therefore regulated independently. The ratios of tartaric acid to malic acid accumulation differ with variety, as does the total level of acid accumulation. The basis for these phenomena remains unknown, and the extent to which viticultural practices may alter particularly these ratios, largely untested. In addition, the genes involved in tartaric acid metabolism are largely unknown, and grapes are one of the few fruits that accumulate this acid. The genes involved in malic acid metabolism are essentially known, however the most important regulatory points with respect to environmental influence are unknown.

As acidity can greatly affect berry flavour, and is essential for a successful ferment, malic acid losses as a result of hot weather conditions can be severely detrimental to the quality of the fruit and wine. Tartaric acid however, seems impervious to elevated temperatures. To counter losses of malic acid associated with ripening and during winemaking, winemakers add tartaric acid to juice, must and finished wine. Discussions with winemakers indicate expenditure on tartaric acid during vintage 2009 in the Riverland of SA between $20 and $30 per tonne of grapes crushed; in hotter vintages, this can approach $50 per tonne (a total cost approaching $19 million for the Riverland alone). The present project builds on earlier research, in which grapevine genes involved in tartaric acid synthesis were identified and characterised, along with a complex array of genes and enzyme activities associated with metabolism of malic acid and of ascorbic acid, the precursor of tartaric acid.

Recent modelling data suggest there will be increases in seasonal mean temperatures across many of the grape growing regions of Australia. This presents the possibility for significant impacts on vine biology (such as total growing season length and sink:source relations) as well as potentially resulting in modified composition of grapes at harvest. Grape composition has major implications for wine quality, and shortfalls in key berry attributes such as acid levels must be addressed if quality wine is to be produced. Serious implications are expected to arise due to altered acid levels and thus likely additional costs to winemakers in the form of increased tartaric acid additions at crush. Our present knowledge of the control of berry acidity, and of the responses of different cultivars to increases in growing season temperatures, is inadequate for appropriate management of the problem in the future. Grapevine responses to severe temperature events are dependent upon developmental

timing and management practices, but remain largely uncharacterised at the metabolite, biochemical or molecular levels. Greater understanding of berry acid metabolism under such conditions will enable industry to make informed decisions concerning the most appropriate varieties for future use (in cultivation and as germplasm for varietal improvement), in addition to permitting development of predictive tools for acid management in winemaking.

4. Project Aims and Performance targets

The two main practical aims as outlined in the Executive Summary were to:

1) Identify important regulatory junctions of malic acid metabolism that may be targeted to reduce acid losses during hot periods of the season.
2) Discover new genes involved in the largely uncharacterised tartaric acid biosynthesis pathway, such that tartaric acid production may be manipulated to control acidity in the berry regardless of seasonal temperature.

To address these aims, a number of smaller aims and outputs will be addressed, as outlined below:

Aim 1: To show how grapevines respond to growth under warming temperatures and to extreme temperature events with respect to organic acid metabolism.

Output 1: Field experiments employing elevated temperature strategies to determine the effect of high temperature on organic acid levels and metabolism in industry-relevant settings with field-grown vines.

Output 2: Controlled-environment experiments to determine the effect of high temperature on organic acid levels and metabolism under precisely controlled temperature, light and humidity conditions with potted vines and isolated tissues.

Aim 2: To examine the variability of responses to warming temperatures in selected cultivars and to develop predictive tools for acid management strategies to mitigate the problems associated with low acid levels in musts.

Output 3: Transcript and metabolite profiles of cultivars differing in organic acid developmental accumulation and degradation patterns.

Output 4: Effect of temperature on cytosolic pH and malic acid concentration in various red fruit cultivars.

Aim 3: Characterise key steps in TA synthesis to identify potential control points that may be targeted for increased berry organic acid levels.

Output 5: Enzymatic characterisation of tartaric acid biosynthesis pathway candidate genes.

Output 6: Grapevines with altered expression of genes involved in malic acid and tartaric acid biosynthesis and catabolism.

Output 7: Metabolic analysis of berries naturally lacking tartaric acid.

Output 8: Exploring grapevine leaves as a model for berry organic acid metabolism.

Aim 4: To develop models obtained from whole-plant studies that describe the metabolism of tartaric and malic acids during grape berry development.

Outputs 2, 3 and 6: Metabolic and transcript maps developed as models for malic acid and tartaric acid metabolism in response to elevated temperatures and between cultivars that differ in organic acid accumulation during berry development.

Aim 5: To provide to the Australian grape and wine industry with knowledge to permit the future selection of germplasm with the capability to yield fruit with the desired acid compositional profiles when grown under the higher temperatures predicted to occur in the viticultural regions of Australia in future years.

Outlined in Section 7: Outcome/Conclusion, and Section 9: Appendix 1: Communication.

5. Method

Aim 1: To show how grapevines respond to growth under warming temperatures and to extreme temperature events with respect to organic acid metabolism.

Field experiments employing elevated temperature strategies were used to determine the effect of high temperature on organic acid levels and metabolism in industry-relevant settings. Field-based temperature elevation experiments were carried out at SARDI's Nuriootpa Research Station in the Barossa Valley of South Australia (34°S, 139°E), as described previously (Sadras *et al.*, 2012).

Controlled-environment experiments were used to determine the effect of high temperature on organic acid levels and metabolism under precisely controlled temperature, light and humidity conditions with potted vines and isolated tissues. This was achieved through the use of Conviron chambers at The Plant Accelerator, The University of Adelaide Waite Campus, South Australia, as described by Sweetman *et al.* (2014).

Aim 2: To examine the variability of responses to warming temperatures in selected cultivars and to develop predictive tools for acid management strategies to mitigate the problems associated with low acid levels in musts.

Field-based comparisons of numerous cultivars for organic acid profiles were carried out using vines from the Coombe Vineyard, University of Adelaide Waite Campus, South Australia (35˚S, 139°E). Bunches were tagged at fruit-set and harvested at weekly intervals throughout development and ripening. Organic acid profiling was conducted as previously described (Sweetman *et al.*, 2012) and transcript and metabolic profiling were carried out as described by (Sweetman *et al.*, 2014).

A new method was explored for the estimation of berry cytosolic pH *in vivo*. This involved the use of a pH-sensitive fluorescent stain, 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Sigma-Aldrich). First, infiltration of the stain into the cytoplasm and nucleus of grape berry cells was confirmed by visualisation of stained berry slices using fluorescent confocal microscopy. To estimate berry cytosolic pH in experimental samples, a 96-well spectrophotometric plate reader was used to measure the fluorescence of berry slices at one emission wavelength (540 nm), with exposure to two separate excitation wavelengths (440 and 485 nm), within an opaque black plate. As the fluorescence of BCECF at 540 nm is pH-dependent when excited at 485 nm, but

pH-independent when excited at 440 nm, emission intensity ratios of 485/440 are representative of pH of the cytosol and unaffected by small differences in rates of infiltration, tissue thickness, etc. Berries were sliced in petri dishes containing a sucrose solution made to the osmolality observed in a subset of berry samples earlier the same day, rinsed with more sucrose solution, then transferred to a well of a plate, containing 50 µL of fresh sucrose solution. BCECF-AM stain (50 µL) was added to tissue slices at a final concentration of 0.4 µg/mL and incubated at room temperature in the dark for 30 minutes, allowing sufficient time for infiltration of the stain into the cell cytosol. With longer incubations, a decline in pH was observed as the stain reached the acidic vacuoles. An *in vivo* calibration curve was generated using extra berry slices pre-incubated in sucrose-containing pH- buffers ranging from pH 6.5 to pH 8.0, comprised of 50mM MES, 50 mM HEPES, 200 mM KCl, 0.1 mM $CaCl_2$ and pH-adjusted using NaOH. Nigericin, an ionophore for H^+ and K^+, was also added to a final concentration of 50 µg/mL to enable equilibration of the grape berry cell pH with that of the surrounding pH buffer. Calibration curves were generated using the 485/440 ratios and used to estimate pH of the unknown samples.

Aim 3: Characterise key steps in TA synthesis to identify potential control points that may be targeted for increased berry organic acid levels.

Methods for the enzymatic assays of specific recombinant candidate TA biosynthesis gene products are described in the results/discussion sections. GC/MS methods for identification of assay products of the 2-keto-L-gulonate reductase were carried out according to derivatisation and analytical measurements of Sweetman *et al.* (2014), and the NIST library.

The generation of transgenic microvines (Chaib *et al.*, 2010) containing altered expression constructs for TA- and MA-metabolising gene candidates was carried out using protocols from within Mark Thomas' laboratory, using agrobacterium infiltration of microvine "V6" embryos isolated from embryogenic calli. Overexpression constructs were developed using restriction-ligation cloning with pCLB1301 and Gateway recombination with pBMTh2 (binary plasmid backbones generated in Dr Mark Thomas' lab). RNAi constructs were developed using Gateway recombination with pHellsgate12. All constructs confer hygromycin resistance *in planta* for selection, and the overexpression constructs also contained a GFP indicator. The following genes were targeted for overexpression: *VvPepc2* (XM_002280806; a phosphoenolpyruvate carboxylase gene lacking the phosphorylation regulation site: the over-expression of this gene has the potential to increase malic acid levels); *VvStop1* (XM_002270160; a transcription factor that potentially regulates genes involved in malic and tartaric acid metabolism); *VvGalur* (NM_001281196; a galacturonic acid reductase gene, involved in the biosynthesis of ascorbic acid, a precursor of tartaric acid: its overexpression has the potential to increase tartaric acid levels); and three *VvLidh* genes (XM_002269900, XM_002269859 and

XM_002267626; encoding L-idonate dehydrogenases. At least one of these *isoforms* of L-idonate dehydrogenase is involved in tartaric acid biosynthesis). The following genes were targeted for a reduction in gene expression by RNAi: *VvNadme2* (XM_002266661; a mitochondrial malic enzyme gene that may be involved in mitochondrial malic acid catabolism); *VvLidh1* (XM_002269900; the most characterised isoform of the L-idonate dehydrogenase gene, likely to be involved in tartaric acid biosynthesis), *VvLidh1and2* (XM_002269900 and XM_002269859; a common region to both genes, with the aim of knocking down transcription of both genes) and *Vv2Kgr* (XM_003632812; 2-ketogulonic acid reductase, a candidate gene for the step in the tartaric acid biosynthesis pathway preceding that catalysed by L-idonate dehydrogenase). A list of primers used to amplify gene inserts for each construct is given in Table A5.1.

Aim 4: To develop models obtained from whole-plant studies that describe the metabolism of tartaric and malic acids during grape berry development.

Leaf organic acid levels were measured throughout development, to explore the possibility of using leaves as a model system for berry organic acid metabolism. Organic acids were extracted and measured according to Sweetman et al. (2011), a method developed for berries which proved to be equally suited for the isolation of organic acids from grapevine leaves.

Metabolic and transcript datasets were pooled manually to develop models for malic acid and tartaric acid metabolism in response to elevated temperatures and between cultivars that differ in organic acid accumulation during berry development.

6. Results/Discussion

6.1. Output 1

Temperature elevation strategies for determining the effect of high temperature on organic acid levels and metabolism in industry-relevant settings, with field-grown vines.

Long-term treatments with small temperature differentials (i.e. year-round warming by 2-4 °C during the day) were applied to Shiraz grapevines in the field and the effects on malic acid levels were determined.

Data were collected over two seasons. When plotted against chronological time (days after flowering), all cultivars demonstrated accelerated patterns of malic acid accumulation and loss (by approximately 5-7 days) when exposed to elevated temperatures (Figure 1a,c,e,g). However, when plotted against the cumulative growth degree days (i.e. the sum of daily temperatures exceeding 10°C) the malic acid concentration curves of control and heated vine berries were almost perfectly overlaid (Figure 1b,d,f,h). Therefore, while this demonstrated that there were no other confounding effects of the experimental apparatus, there was no direct effect of the temperature increase on malic acid concentration in any cultivar. While, on the same calendar day berries from heated vines contained lower concentrations of malic acid than berries from control vines, this was due to advanced development of the heated fruit rather than a direct effect on malic acid metabolism. This was also demonstrated when malic acid concentrations were plotted against TSS (Figure 2).

In a previous study a similar method was used to heat vines in the field by 2-3°C during the day, however treatments were applied for three week periods at specific berry developmental stages rather than throughout the whole year, over multiple seasons. In that experiment, heating of vines at the pre-véraison berry stage did not alter berry malic acid levels at harvest, however heating during véraison or ripening stages led to significantly lower levels of malic acid at harvest (at comparable °Brix). These data were published along with data from the present project (Sweetman *et al.*, 2014). The accelerated development of berries exposed to continuous elevation of daytime temperature by 1-2°C throughout the entire year in the present study therefore attenuated the effects of increased temperature on malic acid levels at harvest.

As these treatments had no specific effect on malic acid levels, an alternative approach was taken, whereby controlled-environment chambers were used to simulate the shorter and more extreme heating events that had been shown to affect malic acid levels in a previous study. Results from this controlled-environment study are given in Output 2.

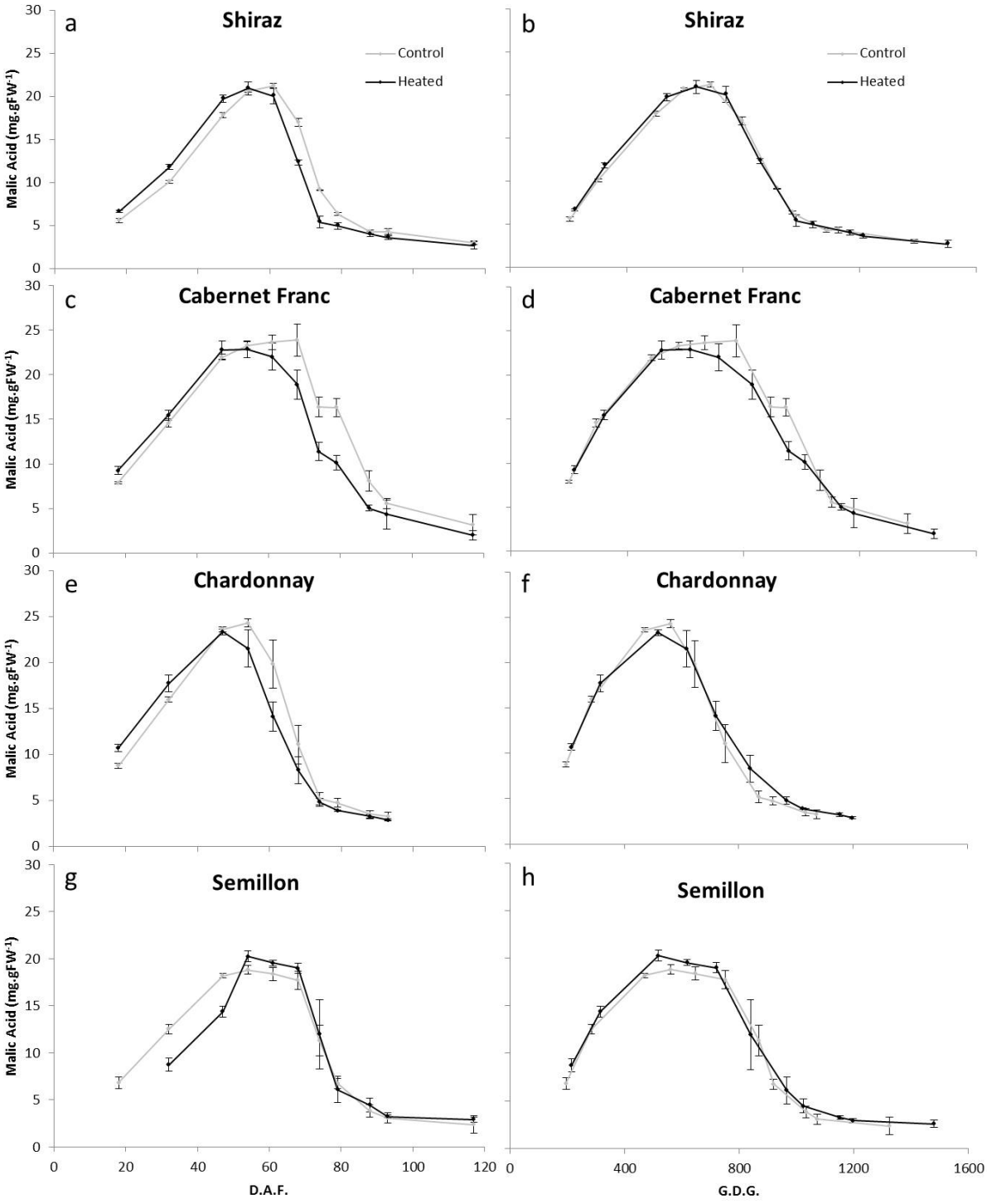


Figure 1: Effect of long-term, small-degree daytime temperature elevation treatments under field conditions on malic acid levels. Malic acid concentration presented in relation to Days After Flowering (D.A.F.) (left) and Growth Degree Days (G.D.D.) (right) for Shiraz, Cabernet Franc, Chardonnay and Semillon cultivars under control and heated conditions in the 2010-11 season. The heat treatment involved use of open-top chambers with temperature elevation by approximately 1-2°C during the day over three consecutive years (from October 2009), as described by Sadras et al. (2012). (n = 3 ± S.E.M.).

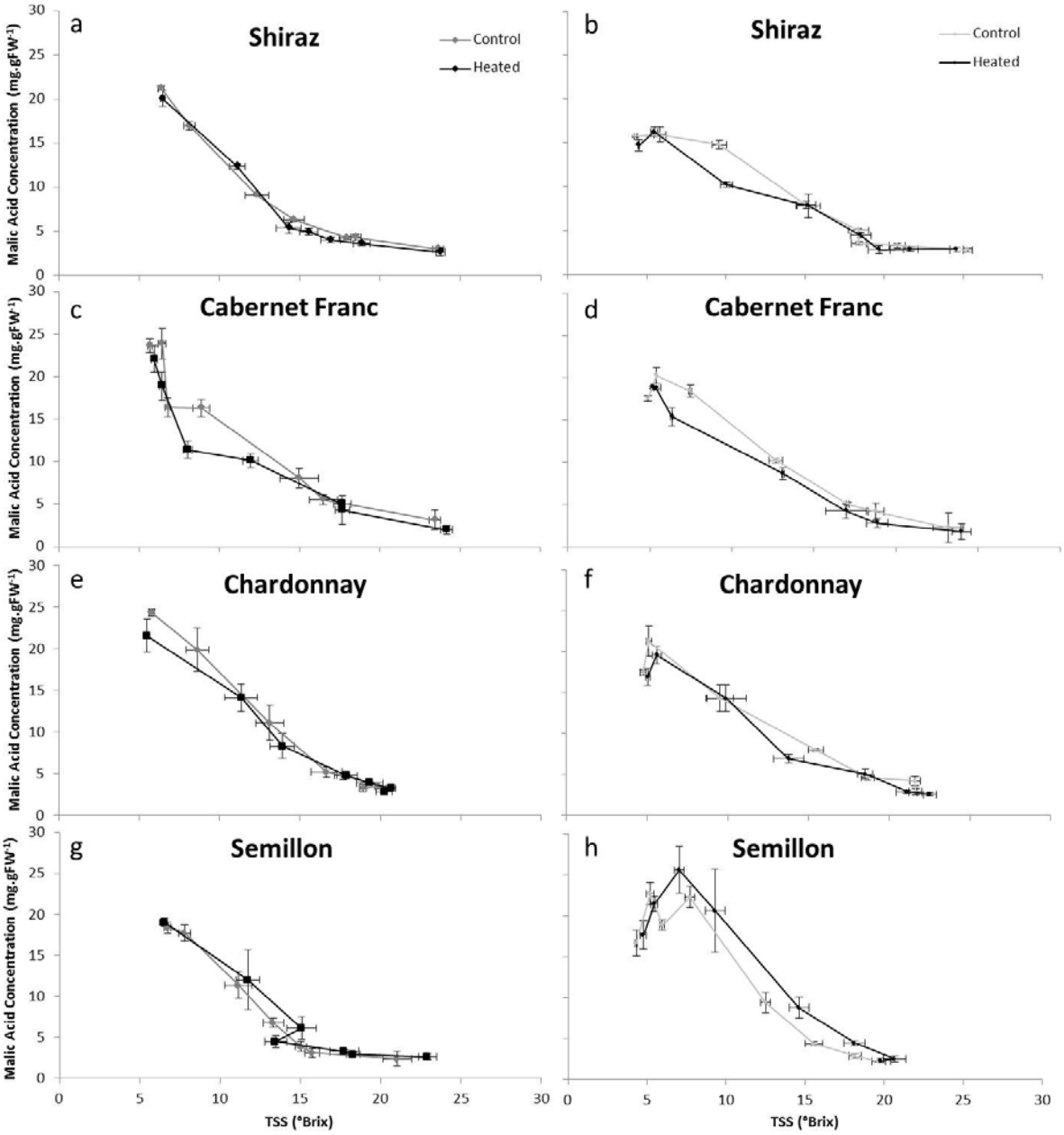


Figure 2: Effect of long-term, small-degree daytime temperature elevation treatments under field conditions on malic acid levels over two berry developmental seasons. Season 2010-11 (left) and Season 2011-12 (right) for Shiraz, Cabernet Franc, Chardonnay and Semillon cultivars under control and heated conditions. The heat treatment involved use of open-top chambers with temperature elevation by approximately 1-2°C during the day over three consecutive years (from October 2009), as described by Sadras et al. (2012). Malic acid levels were plotted against TSS (n = 3 ± S.E.M.).

6.2. Output 2

Controlled-environment experiments to determine the effect of high temperature on organic acid levels and metabolism under precisely controlled temperature, light and humidity conditions with potted vines and isolated tissues.

Short-term treatments with large temperature differentials were applied to potted grapevines at specific developmental stages using controlled environment chambers, and the effects on malic acid levels were determined. These data were used for comparisons with short-term field temperature experiments conducted in a previous study.

Potted vines were treated under identical controlled environment conditions in two separate seasons (2011-12 and 2012-13). Shiraz was selected for use in this study, as it is the most common red winemaking cultivar in Australia and the subject of a similarly structured field-based experiment (Sweetman *et al.*, 2014). Vines exposed to elevated temperatures at either pre-véraison or véraison stages had berries of smaller fresh weights in both seasons. The pre-véraison treatment led to a small, temporary increase in malic acid concentration that disappeared by véraison (Figure 3). The véraison treatment led to lower levels of malic acid during ripening; an effect still noticeable in ripe fruit (Figure 3). Heated fruit also had slightly higher TSS, suggesting advanced maturity of these berries relative to control berries. Therefore the lower levels of malic acid observed in ripening fruit of heated vines could be due to advanced maturity of these fruit relative to control vines. However, when malic acid levels were plotted as a function of TSS to align the two treatment groups based on maturity, the berries of heated vines demonstrated lower levels of malic acid, therefore a direct effect of elevated temperature on malic acid loss was observed in ripening grapes (Figure 3). Tartaric acid levels showed a slight decrease in heated berries when expressed per berry but not per gram fresh weight, due to the smaller berry weights (Figure 4). Overall, the environmental chambers could satisfactorily simulate a heatwave in the field, providing tissue for further analysis of the mechanisms of malic acid loss under controlled conditions.

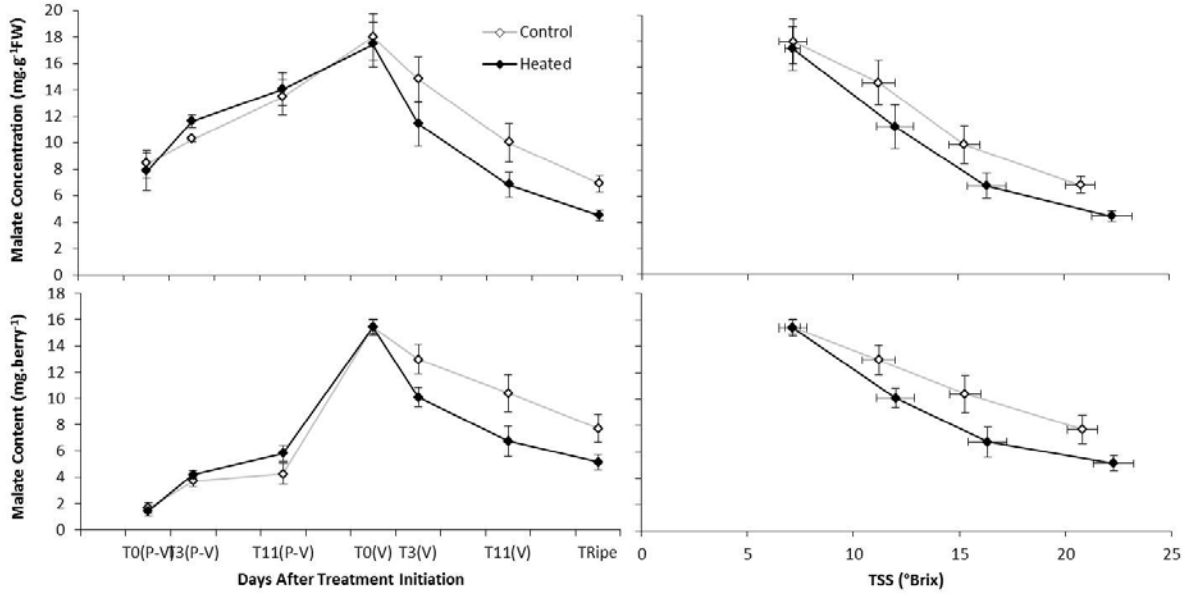


Figure 3: Effect of short-term, high-degree temperature elevation treatments under controlled conditions on malic acid levels. Each graph represents data pooled from two seasons, 2011-12 and 2012-13, presented in chronological time (left) and relative to TSS (right), and presented as mg per gram fresh weight (top) or mg per berry (bottom). Potted vines were kept in "control" or "heated" conditions within controlled-environment chambers. Temperature elevation periods lasted 11 days, reaching 10°C above control temperatures during the day and 5°C above control temperatures during the night. Vines were treated at pre-véraison (P-V) and véraison (V) stages. (n = 4 ± S.E.M.).

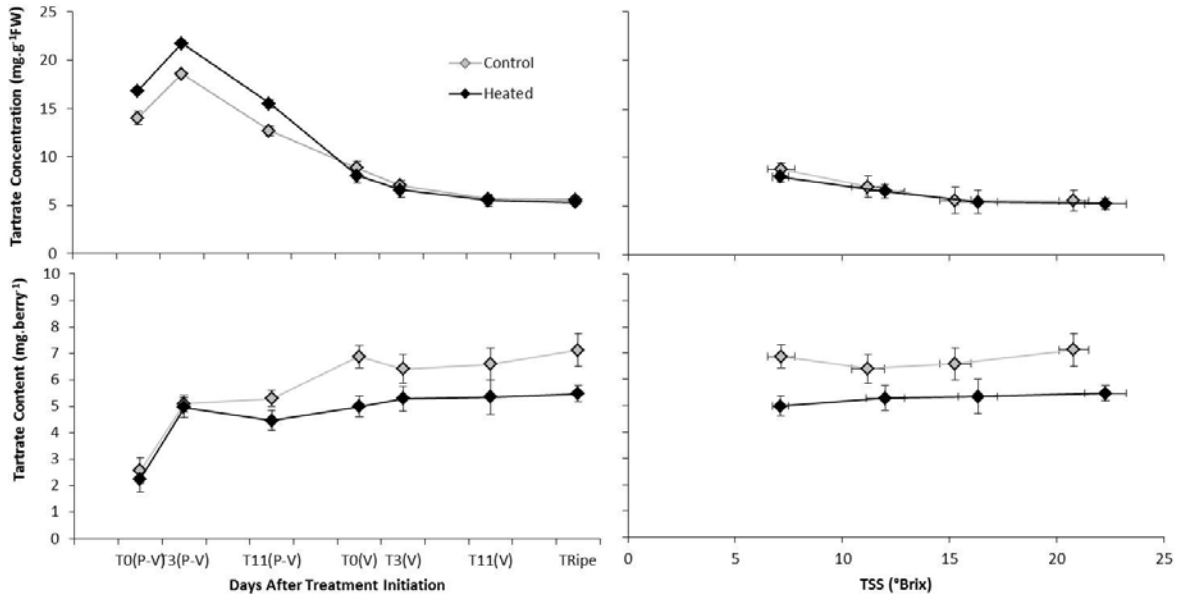


Figure 4: Effect of short-term, high-degree temperature elevation treatments under controlled conditions on tartaric acid levels. Each graph represents data pooled from two seasons, 2011-12 and 2012-13, presented in chronological time (left) and relative to TSS (right), and presented as mg per gram fresh weight (top) or mg per berry (bottom). Potted vines were kept in "control" or "heated" conditions within controlled-environment chambers. Temperature elevation periods lasted 11 days, reaching 10°C above control temperatures during the day and 5°C above control temperatures during the night. Vines were treated at pre-véraison (P-V) and véraison (V) stages. (n = 4 ± S.E.M.).

6.2.1. Transcript Profiles

Berries from the véraison heat treatment were used for the analysis of transcript levels at "véraison" (three days after the initiation of the véraison treatment) and "ripening" (11 days after the initiation of the véraison treatment). Therefore the berries used for the "véraison" sample collection had been exposed to a short-term increase in temperature, whereas the berries used for the "ripening" sample collection had been exposed to a longer period of elevated temperature. Transcript levels of a gene encoding a heat shock protein, *VvHsp18.2*, increased up to four-fold in berries from heated vines compared to berries from control vines, confirming that the increase in temperature was sufficient to elicit a stress response (Figure 5). Transcript levels of genes encoding L-idonate dehydrogenase, involved in tartaric acid biosynthesis in grapes, were not responsive to elevated temperature conditions. This was not surprising, considering that the levels of tartaric acid were not affected by the elevated temperature treatment. However, transcript levels of genes suspected to be involved in the regulation of malic acid metabolism did show various responses to the elevated temperature treatments. Some gene transcripts were affected early during the treatment, some later and some consistently at both "véraison" and "ripening" time-points. A number of other transcript levels remained unchanged. Only the genes or gene families that responded to elevated temperature treatments are presented.

Transcript levels of genes encoding NADP-dependent malic enzymes (situated in the cytosol and chloroplast) were unaffected by temperature (Figure 6). However a gene encoding an NAD-dependent malic enzyme of the mitochondria, *VvNadme1* showed increased transcript levels in heated berries (two-way ANOVA, $p = 0.047$) (Figure 6). *VvMmdh1*, a mitochondrial malate dehydrogenase gene, was up-regulated in a similar manner although not significantly (two-way ANOVA, $p = 0.188$) (Figure 7). Therefore there is increased potential for mitochondrial malic acid metabolism in berries of heated vines.

Two genes putatively encoding cytosolic malate dehydrogenases (*VvCmdh1* and *VvCmdh2*) were also significantly up-regulated (two-way ANOVA, $p = 0.046$ and $p = 0.029$ respectively) in response to elevated temperature in véraison and ripening berries (Figure 7), suggesting that not only the mitochondrial pathways, but perhaps cytosolic malic acid metabolic pathways have the capacity to increase in response to elevated temperature in grape berries. However, a third putative cytosolic malate dehydrogenase gene, *VvCmdh3*, demonstrated significant down-regulation in response to elevated temperature in véraison and ripening berries (two-way ANOVA, $p = 0.017$). Therefore some isoforms are favoured over others during elevated temperatures, possibly eliciting tissue- or cell-specific responses, or conferring different kinetic properties in the resulting MDH protein. Taureilles-

Saurel *et al.* (1995) demonstrated that although the kinetic properties of "cytosolic" and "mitochondrial" MDHs were quite similar, the mitochondrial MDH activity may operate more efficiently than cytosolic MDH at higher temperatures. Individual genes that may encode unique MDH isoforms within these compartments have not been characterised separately. Therefore it is unknown whether the enzymes corresponding to the other two cytosolic MDH genes (*VvCmdh1* and *VvCmdh2*), which both showed increased transcript levels with heating, may represent the majority of MDH activity in the cytosol, or whether the decrease in *VvCmdh3* transcript level may result in a significant decrease in MDH activity *in vivo*.

A putative phosphoenolpyruvate carboxylase gene (*VvPepc2*) showed an 80% decrease in transcript levels in véraison berries exposed to elevated temperatures (Figure 8). In conjunction with the decreased *VvCmdh3* transcript levels mentioned previously, the synthesis of malic acid from glycolytic intermediates may therefore be impeded at higher temperatures. In a paper that we published during the completion of this work, malic acid levels in heated grape berries showed some correlation with PEPC activity in Shiraz berries at véraison (Sweetman *et al.*, 2014), therefore this is a likely candidate for improving the levels of malic acid during warmer temperatures.

To investigate the potential for malic acid transport within the cell, an aluminium-activated malate transporter-like gene, *VvAlmt9*, previously found to assist the import of malic acid into the vacuole (De Angeli *et al.*, 2013), was investigated. Transcript levels of this gene decreased with elevated temperature (two-way ANOVA, $p = 0.038$), suggesting a decrease in the capacity for transporting malic acid into the vacuole in heated berries at véraison, a time when cellular de- compartmentalisation may be occurring (Figure 9). Interestingly, transcript levels of a putative tonoplast dicarboxylate transporter, *VvTdt1*, were significantly (two-way ANOVA, $p = 0.045$) up- regulated in response to elevated temperature in véraison and ripening berries (Figure 9). Taken together, a change in the compartmentalisation of malic acid from metabolic enzymes in the cytosol and mitochondria could be the underlying, rate-limiting step in malic acid degradation during ripening. This is not the first time that such a hypothesis has been proposed (Terrier *et al.*, 2001), and further investigation is required *in vivo* to determine whether the activities of these transporters in the berries respond to changes in environmental conditions. Transcript levels of vacuolar pyrophosphatase genes, which generate proton gradients in the vacuole that help to drive malic acid accumulation, did not appear to be affected by the elevated temperature treatment (Figure 9).

Transcript levels of the gluconeogenic enzyme PEP carboxykinase *VvPepck,* showed minor decreases in response to the elevated temperature treatment (Figure 10), although not significant (two-way

ANOVA, p = 0.513). Our earlier study with field-grown Shiraz vines showed a similar non-significant decrease in *VvPepc2* transcript level with elevated day temperatures at véraison and a significant decrease in PEPC activity (Sweetman et al. 2014). *VvPpdk*, which putatively encodes a pyruvate,Pi dikinase enzyme that may also be involved in gluconeogenesis showed no changes in transcript level with elevated temperature (figure 10), again consistent with the field-based experiment (Sweetman *et al.*, 2014).

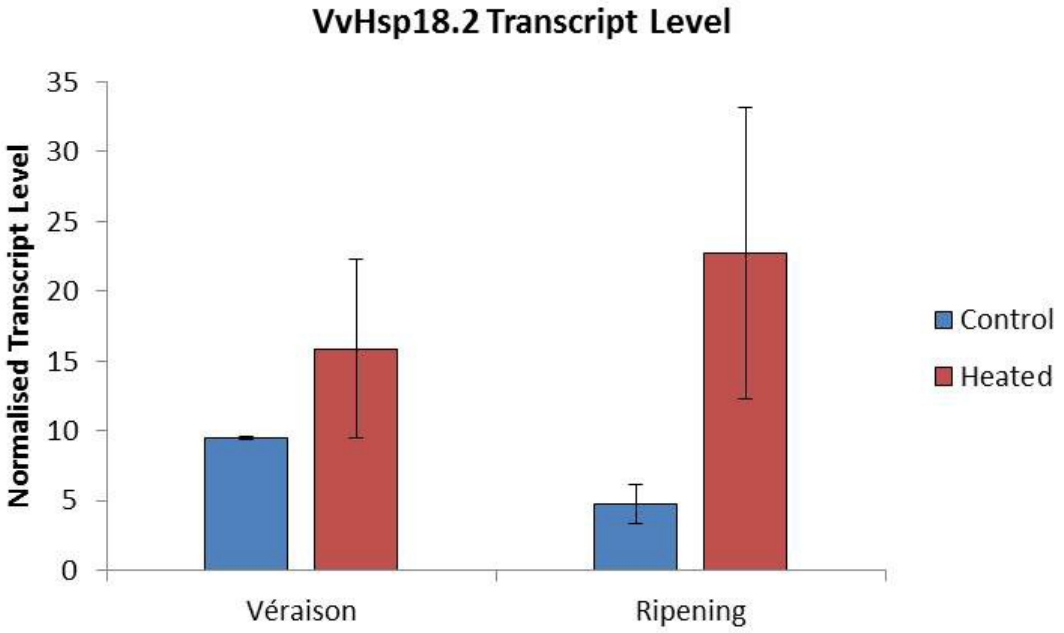


Figure 5: Transcript levels of heat-shock protein gene, *VvHsp18.2* in response to elevated temperature. Samples were collected during véraison (three days after treatment initiation) and ripening (11 days after treatment initiation) stages of berry development (n = 4 ± S.E.M.)

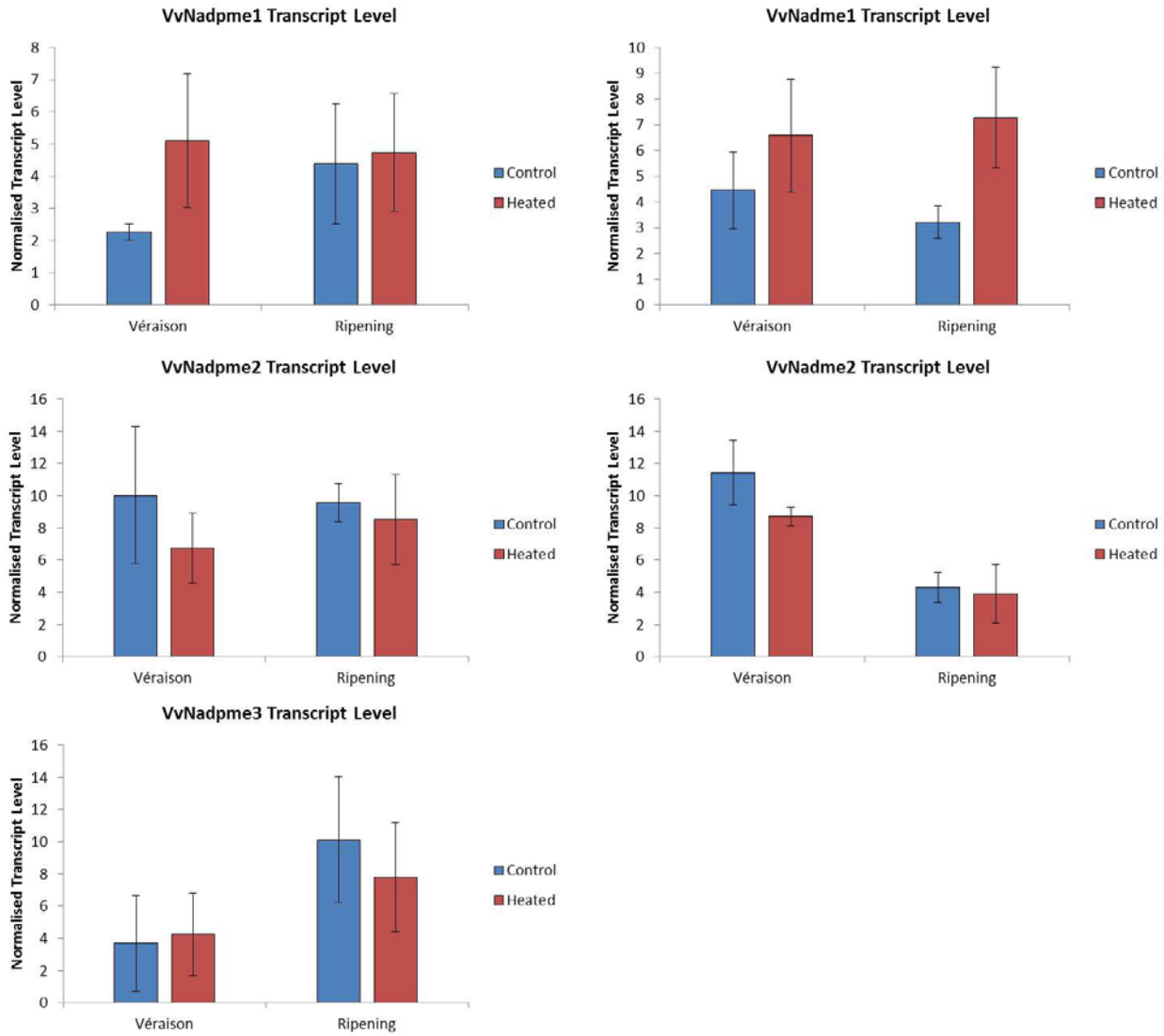


Figure 6: Transcript levels of malic enzyme genes in response to elevated temperature.
Cytosolic/chloroplastic (NADP-dependent) ME genes are presented on the left, mitochondrial (NAD-dependent) ME genes are presented on the right. Samples were collected during véraison (three days after treatment initiation) and ripening (11 days after treatment initiation) stages of berry development (n = 4 ± S.E.M.)

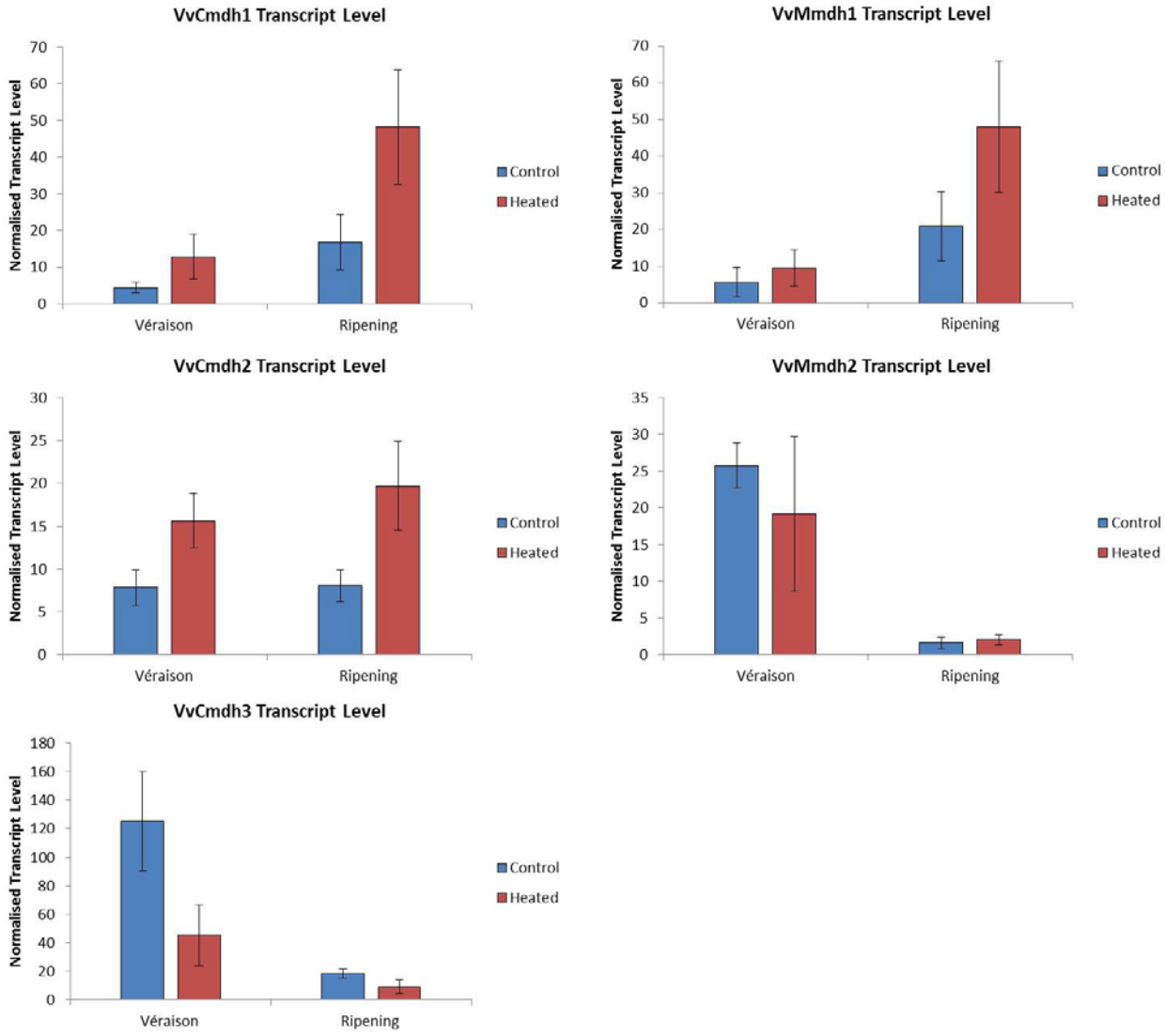


Figure 7: Transcript levels of malate dehydrogenase genes in response to elevated temperature. Cytosolic MDH genes are presented on the left, mitochondrial MDH genes are presented on the right. Samples were collected during véraison (three days after treatment initiation) and ripening (11 days after treatment initiation) stages of berry development (n = 4 ± S.E.M.)

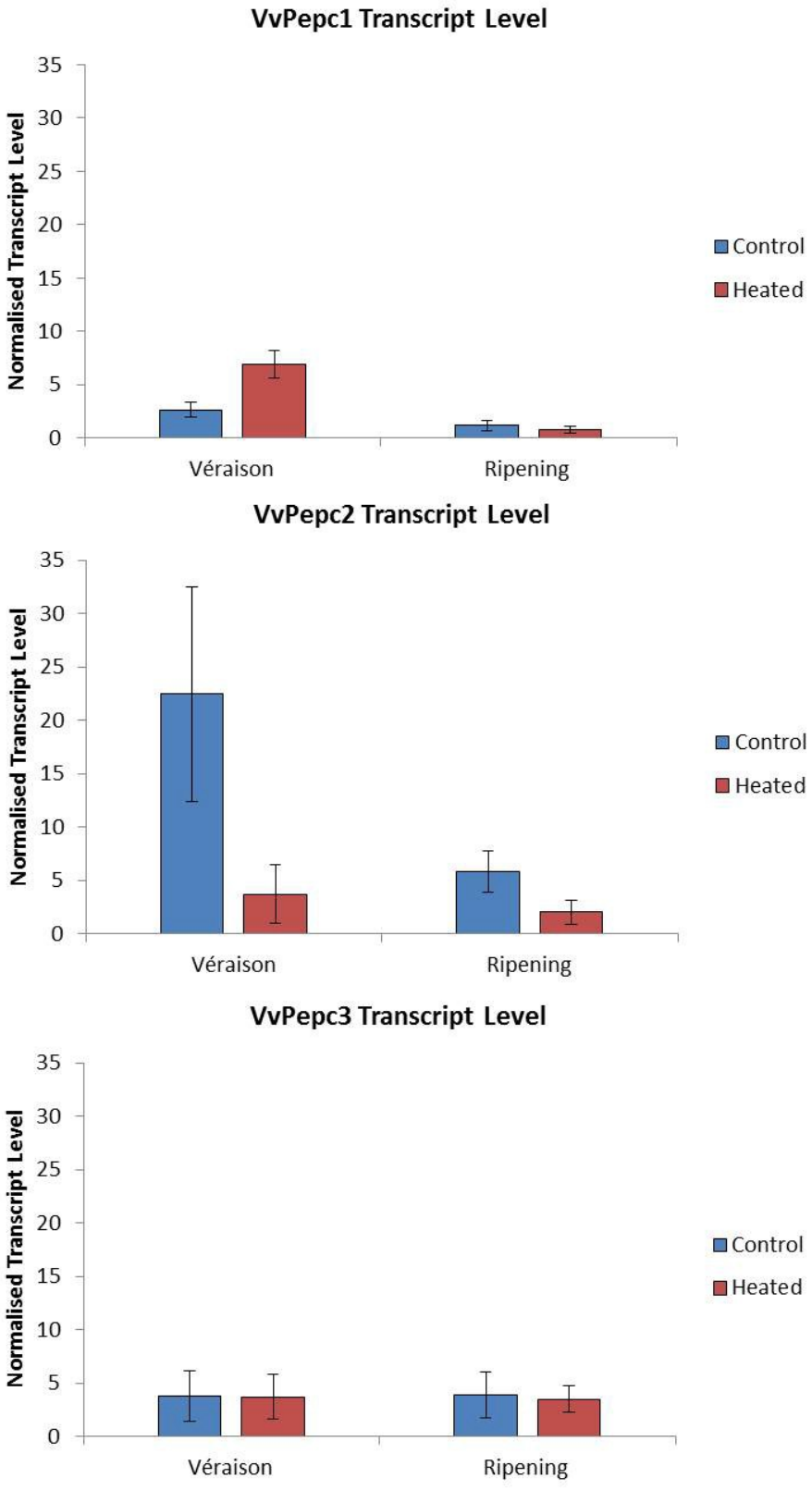


Figure 8: Transcript levels of phosphoenolpyruvate carboxylase genes in response to elevated temperature. Samples were collected during véraison (three days after treatment initiation) and ripening (11 days after treatment initiation) stages of berry development (n = 4 ± S.E.M.).

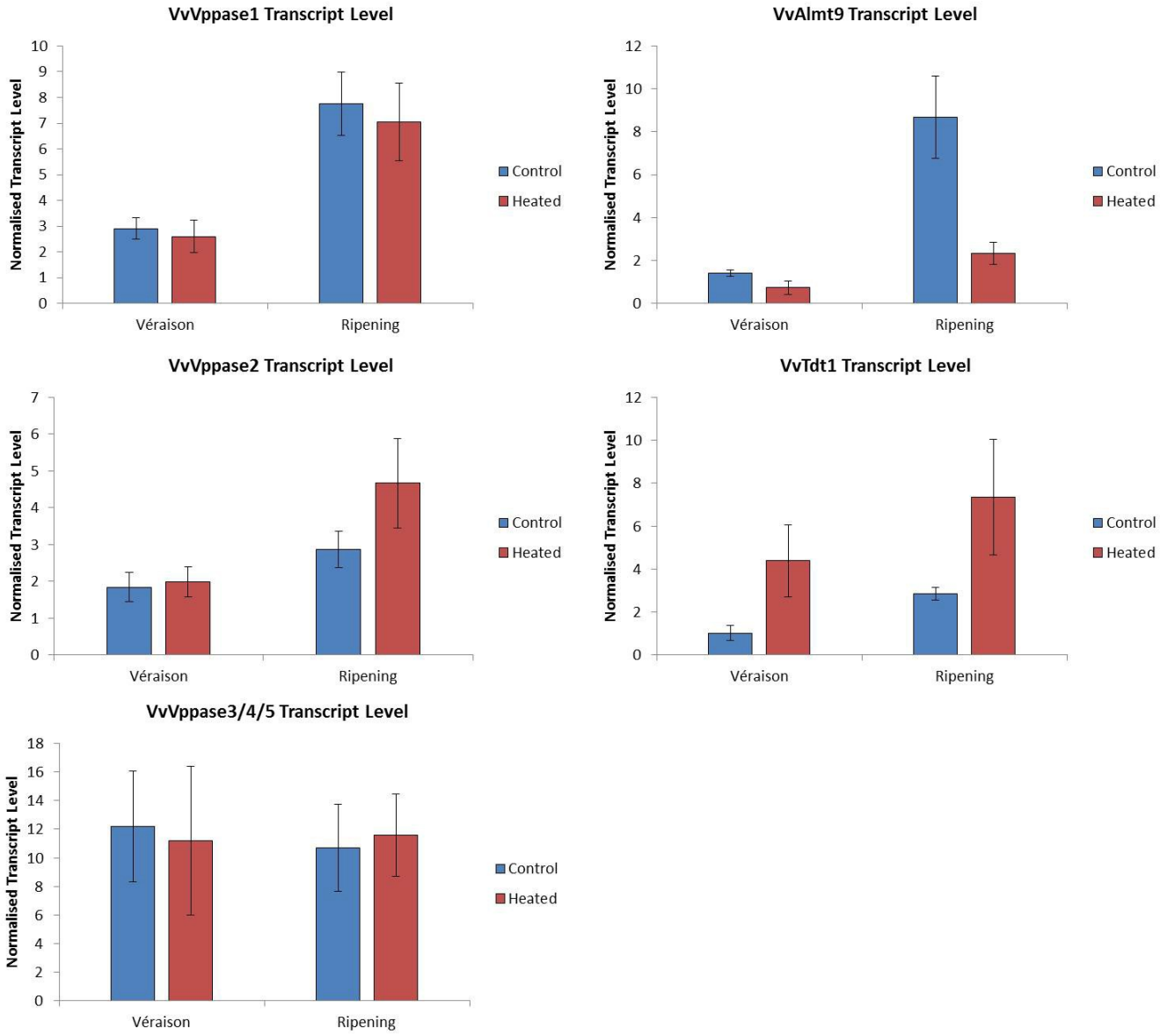


Figure 9: Transcript levels of vacuolar transporter genes in response to elevated temperature. Vacuolar pyrophosphatase genes are presented on the left, malic acid transporter genes are presented on the right. *VvVppase3/4/5* refers to genes that could not be assayed individually due to high sequence identity at the nucleotide level. Samples were collected during véraison (three days after treatment initiation) and ripening (11 days after treatment initiation) stages of berry development (n = 4 ± S.E.M.)

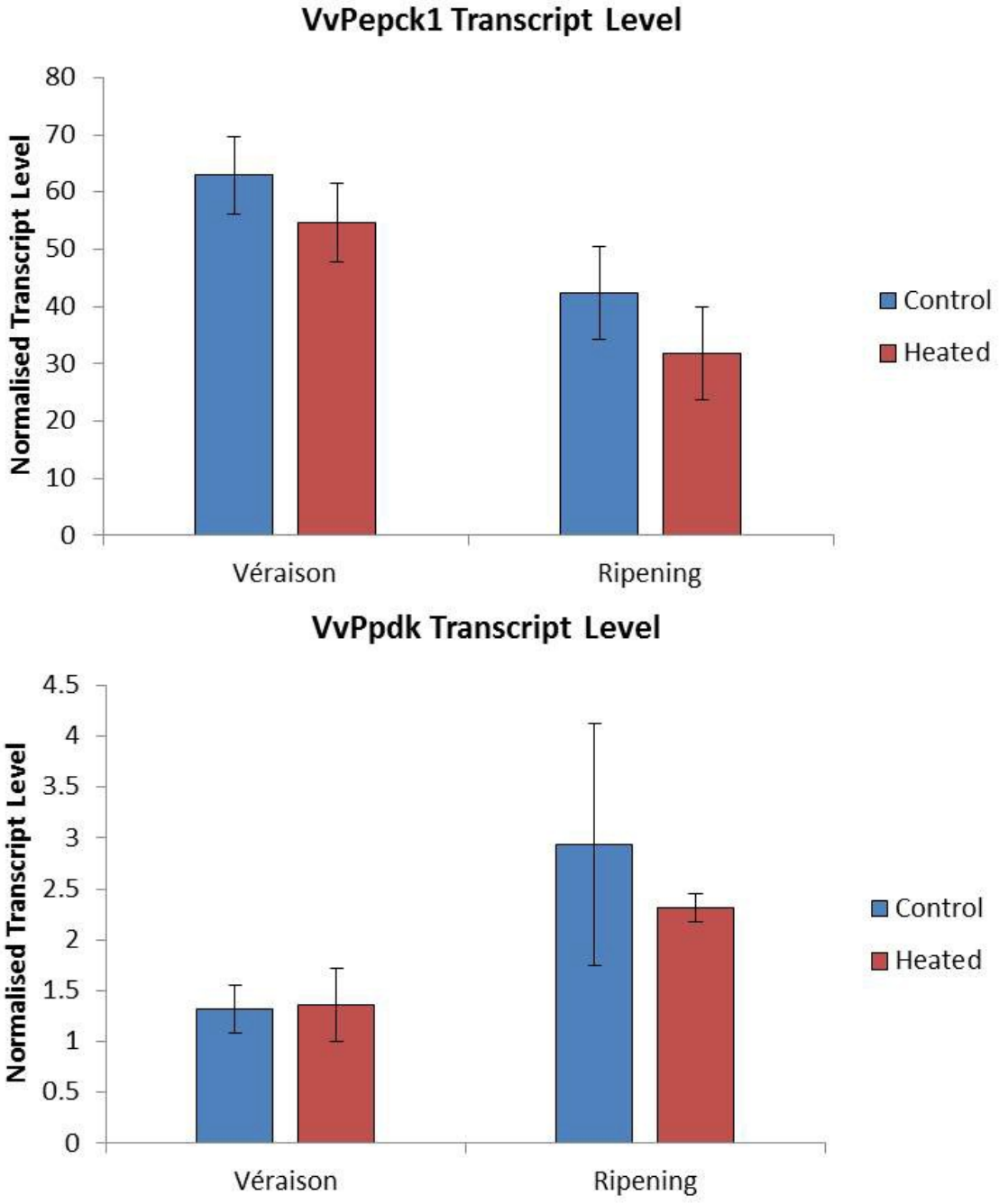


Figure 10: Transcript levels of genes involved in gluconeogenesis in response to elevated temperature. Samples were collected during véraison (three days after treatment initiation) and ripening (11 days after treatment initiation) stages of berry development (n = 4 ± S.E.M.)

6.2.2. Metabolite Profiles

Berries from the véraison heat treatment were also used for a metabolomic analysis, conducted during a GWRDC-supported visit to the James Hutton Institute, Dundee, Scotland, UK in May, 2013. Berries at multiple time-points during and after the véraison elevated temperature treatment were used in this analysis. The first two time-points represent those used for transcript level analysis (i.e. three and 11 days after the initiation of the treatment) and the subsequent time-points once vines had returned to control temperatures during ripening. Metabolite data suggested that the relative degree of malate loss from berries exposed to elevated temperatures, although significant, was small when compared to relative changes in amino acid levels, which generally increased in heat-treated fruit. This finding needs to be developed further, by looking specifically at amino acid metabolism in response to elevated temperatures during fruit ripening. It may be that the catabolism of malic acid is linked to the anabolism of amino acids. For example, the observed increase in transcript levels of mitochondrial and cytosolic malate dehydrogenases as well as a mitochondrial malic enzyme gene may indicate increased conversion of malate to both oxaloacetate and pyruvate. Increased oxaloacetate may have contributed to the observed increase in aspartate in these samples, while increased pyruvate may have contributed to the observed increase in valine, leucine, serine and glycine. Increases in GABA, glutamate, proline and putrescine were also seen, indicating a stress response to the elevated temperature consistent with the increase in transcript level of the Heat Shock protein gene seen in these berries. Transcript levels of two genes putatively encoding glutamate decarboxylase (GAD), an enzyme that converts glutamate to GABA, were unchanged in response to the elevated temperature treatment and therefore transcriptional regulation of this enzyme may not be limiting for GABA biosynthesis.

Altering the nitrogen availability to the vines may also alter the response of malic acid metabolism to temperature stress. That is, making nitrogen more available to the plant earlier during development may increase the capacity for amino acid synthesis while photosynthesis and glycolysis are still occurring at high rates within the berry. If synthesis of these amino acids is not required during véraison and ripening when the berries rely more on malic acid as a carbon source for respiration and central metabolism, there is potential for retaining more malic acid in the vacuole. This hypothesis needs to be tested by exploring the mechanisms of amino acid synthesis during ripening and whether malic acid is a source of carbon skeletons for this. Radiolabelling experiments have already shown that a significant proportion of label from exogenous malic acid could be recovered in amino acid pools, particularly during ripening (Beriashvili and Beriashvili, 1996). Amino acid biosynthesis enzymes in response to altered temperature need to be explored at the transcript, protein and activity level. The

application of nitrogen fertiliser during pre-véraison berry development should also be tested to determine whether increased amino acid biosynthesis occurs as a result, and whether malic acid levels are affected. Previous studies have found that vines with low nitrogen status produced berries with low malic acid levels and high pH (Reynard *et al.*, 2011), while vines fertilised with nitrogen resulted in higher berry malic acid and citric acid levels (Ruhl *et al.*, 1992). As yet the effect of elevated temperature on nitrogen partitioning remains unknown, but is worth exploring.

As mentioned previously, there were a number of changes in transcript levels that may explain the increased loss of malic acid in ripening berries of heated vines, through increased efflux of malic acid from the vacuole (decreased *VvAlmt9* and increased *VvTdt*), a change in the balance of mitochondrial MDH isoforms (increased *VvMmdh1*, *VvCmdh1* and *VvCmdh2* and decreased *VvCmdh3*), decreased glycolytic PEP carboxylase transcript (*VvPepc2*) and increased mitochondrial NAD-dependent malic enzyme (*VvNadme1*). At the activity level, a decrease in PEP carboxylase and an increase in mitochondrial NAD-dependent malic enzyme have also been observed in heated berries. Increased mitochondrial NAD-ME activity suggests an anapleurotic role for malic acid in the TCA cycle that may be necessary if other intermediates are being drawn off for biosynthesis of amino acids and other metabolites. However the metabolism of malic acid alone may be insufficient to cause such a significant increase in the levels of so many amino acids. Glycolytic intermediates may have also contributed, although there was no negative impact on sugar accumulation, and no significant decrease in transcripts of genes involved in gluconeogenic metabolism within this study (i.e. *VvPepck* and *VvPpdk*).

As the increase in levels of numerous amino acids with elevated temperature may be linked to the increased catabolism of malic acid, the minimisation of malic acid losses during extreme heat events may also be linked to amino acid metabolism and nitrogen availability. Identifying cultivars with altered expression patterns or gene sequences of the above-mentioned genes during véraison and ripening and in response to elevated temperatures may assist in the identification of cultivars with altered malic acid accumulation properties. Of particular interest are the tonoplastic transporter genes *VvAlmt9* and *VvTdt1*. Genes and enzymes involved in the biosynthesis of amino acids require further analysis for their role in regulating malic acid levels.

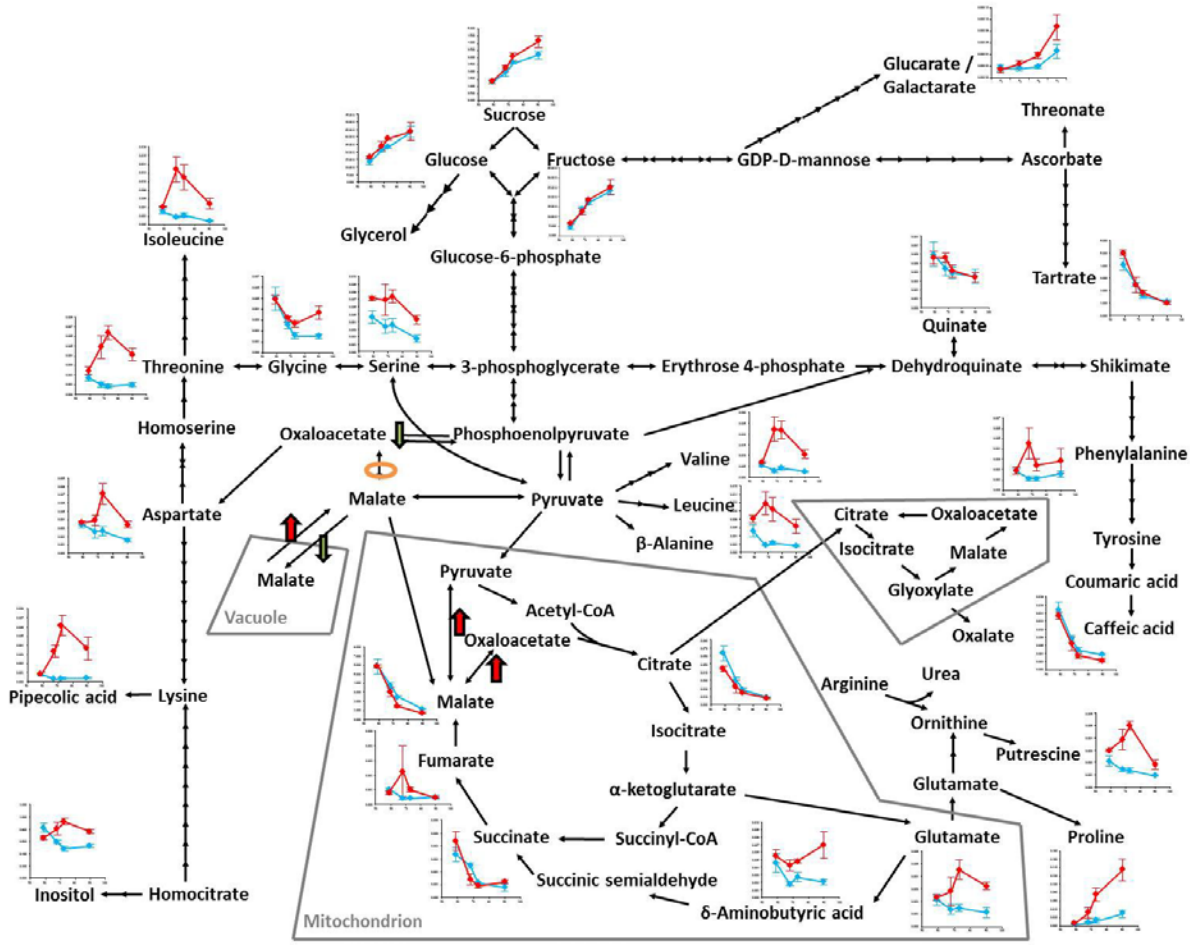


Figure 11: Shiraz controlled-environment elevated temperature treatment transcript and metabolite model

6.3. Output 3

Transcript and metabolite profiles of cultivars differing in organic acid developmental accumulation and degradation patterns.

As the berries of different grapevine cultivars have inherently different levels of organic acids at the time of harvest, the effect of temperature on malic acid levels is also likely to vary between cultivars. As the levels of malic acid are not only determined by the level to which the acid accumulates during early development, but also by the level to which the acid is degraded during ripening, it was important to investigate the pattern of malic acid levels during both development and ripening in cultivars that differ in malic acid at harvest, and therefore provide some insight into how season- long, or short developmental-targeted heating events might affect malic acid levels in these cultivars. It also enabled a transcriptional analysis of genes discussed in Output 2, between cultivars differing in malic acid levels.

6.3.1. Semillon versus Riesling

According to tartrate:malate ratios at harvest, (Kliewer *et al.*, 1967) classified Semillon as an intermediate-malate variety (with a ratio of 1.76:1) and White Riesling as a low-malate variety (with a tartrate:malate ratio of 3.12:1). As commonly used white wine varieties, these cultivars were selected for a more in-depth investigation of malate and tartrate levels during development and ripening in the Coombe vineyard, The University Adelaide. Riesling and Semillon bunches were tagged at anthesis (50% cap-fall) and used for sample collection with high frequency throughout berry development during the 2010-11 season.

Measurements of berry physiology (fresh weight) and composition (total soluble solids) demonstrated the expected patterns of growth and ripening (Figure 12). Semillon berries were significantly larger than Riesling berries from 23 days after flowering.

Tartaric acid levels (per gram fresh weight) were similar in the two cultivars until the ripening stage (stage II), where Semillon tartaric acid exceeded that of Riesling. When expressed per berry, this difference was more pronounced and occurred from early development (Stage I).

Malic acid levels (per gram fresh weight) were similar in the two cultivars until the ripening stage, where the loss of malate was initiated earlier in Semillon than in Riesling. By harvest-ripeness, there was no difference in tartaric acid concentrations between the cultivars. When expressed per berry, Semillon had much higher levels of malate than Riesling berries during development and ripening, but again no difference by the time of harvest. When expressed per berry, the rapid loss of malic acid from Semillon berries between véraison and harvest-ripeness was more pronounced than the loss of malic acid from Riesling berries (Figure 13) due to the higher level of malic acid in Semillon

berries at véraison, which was in turn due to the larger size of the fruit rather than a higher concentration of malic acid per gram fresh weight.

The tartrate:malate ratio at harvest-ripeness was 1.5:1 for Riesling and 2.5:1 for Semillon (Figure 14), consistent with the patterns seen by (Kliewer *et al.*, 1967). However, the difference in these ratios was driven by differences in tartaric acid levels more so than malic acid. Therefore these cultivars were not used in further analysis of malic acid metabolism and cultivars with larger differences in malic acid levels were sought.

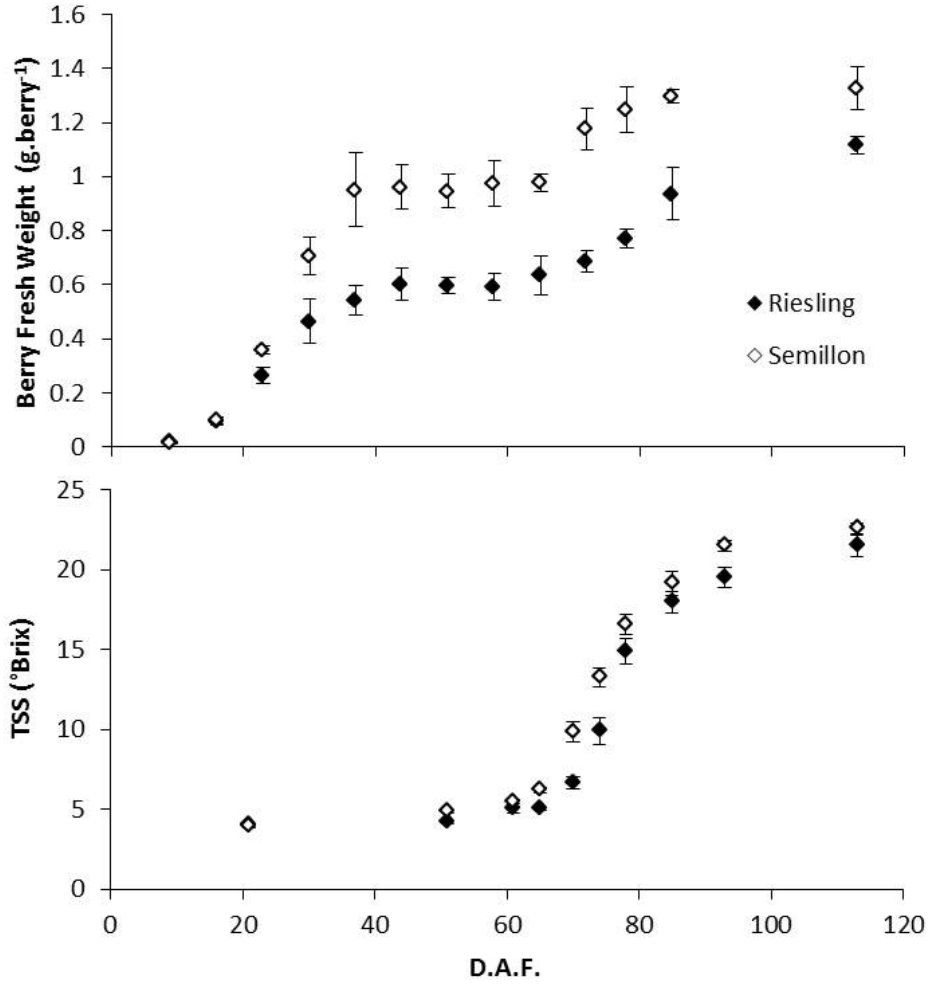


Figure 12: Riesling vs Semillon berry weight and TSS during development and ripening. (n = 4 ± S.E.M.)

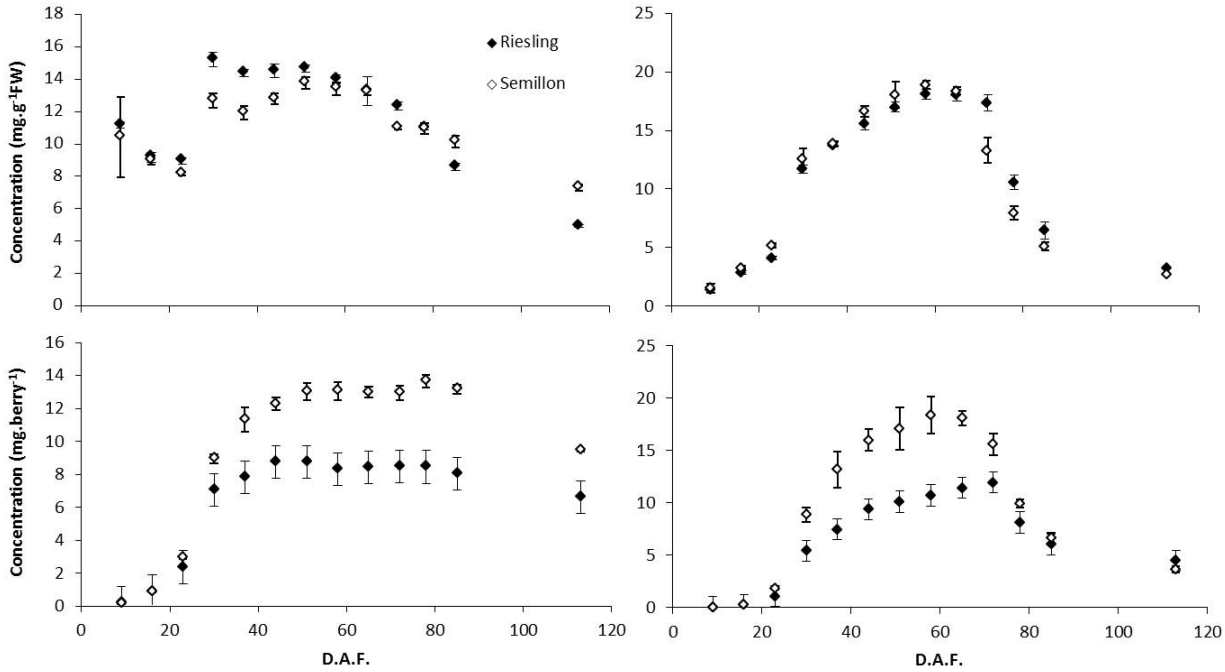


Figure 13: Riesling and Semillon organic acid concentrations during development and ripening. Tartaric acid (left) and malic acid (right) concentrations expressed per gram fresh weight (top) and per berry (bottom) to demonstrate the difference between dilution effects on tartaric acid and active degradation of malic acid (n = 4 ± S.E.M.)

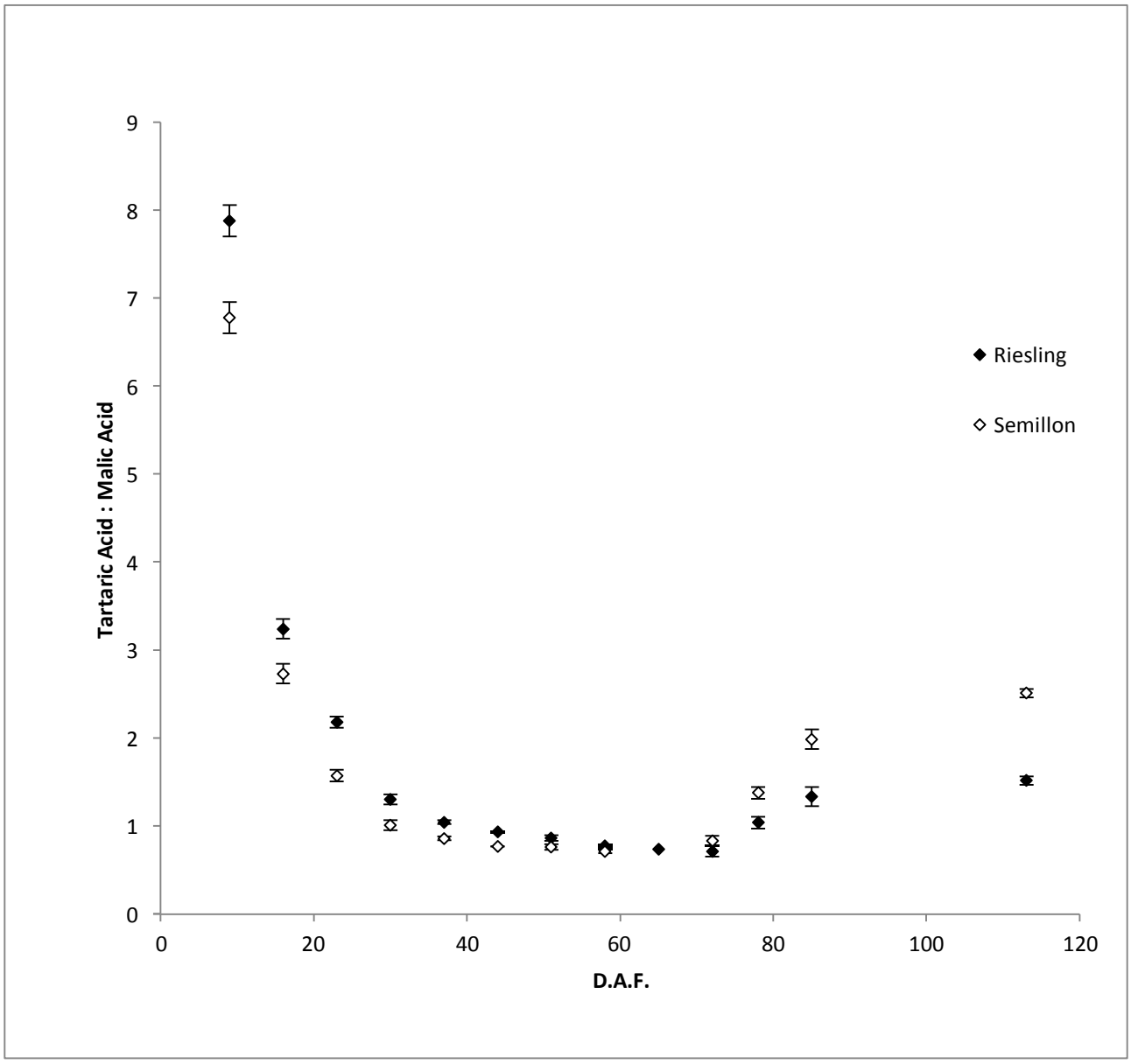


Figure 14: Ratios of tartaric acid and malic acid in Riesling and Semillon fruit during development and ripening. (n=4 ± S.E.M.)

6.3.2. Tinta Cao, Cabernet Sauvignon, Chenin Blanc and Palomino

Cultivars were selected based on their absolute malate levels at harvest rather than tartrate:malate (Kliewer *et al.*, 1967). Two red and two white cultivars were selected. For the whites, Chenin Blanc and Palomino were selected as high- and low- malate cultivars, respectively. For the reds, Tinta Cao and Cabernet Sauvignon were selected as high- and low- malate cultivars, respectively.

A pilot trial was conducted in the 2010-11 season. Samples were collected at véraison and at harvest-ripeness, and tested for malic acid and tartaric acid levels. At harvest the levels of malate were 0.65, 2.82, 3.92 and 6.17 mg/gFW for Palomino, Cabernet Sauvignon, Chenin Blanc and Tinta Cao, respectively. Therefore there was an almost ten-fold difference between the lowest- and highest-malate-containing cultivars. Large differences in tartrate:malate were observed: 6.81, 1.98, 0.92 and 0.68, respectively. This pattern was similar when the data were expressed per berry, and also similar to data from Kliewer *et al.* (1967), who found respective ratios of 3.41, 2.43, 1.05 and 1.08.

Malic acid concentrations in the four varieties at harvest were plotted alongside other varieties that were collected for various other experiments during the 2010-11 season from the Coombe and Nuriootpa Research Station vineyards, to determine how the malic acid levels compared with other cultivars (Figure 15). Palomino was found to contain the lowest level of malic acid of all cultivars tested, and Tinta Cao demonstrated the highest. Chenin Blanc and Cabernet Sauvignon fell into the middle of the dataset. Therefore these four cultivars were deemed to have sufficiently different malic acid concentrations to warrant in-depth investigation. The first aim was to determine which of two possible scenarios was responsible for causing differences in malic acid levels: acid accumulation during berry development or acid degradation during ripening. Also to look at how these cultivars differed in terms of berry composition (particularly amino acid levels) and transcript levels of various genes of interest highlighted in Output 2. To begin with, full developmental profiles of basic berry physiology and composition were compared, including malic and tartaric acid levels. Data were collected during two developmental seasons to determine whether differences in malic acid levels between the cultivars were seasonally dependent. The data are presented for individual seasons (Figures 16 and 17) and for pooled seasons (Figure 18 and Table 1).

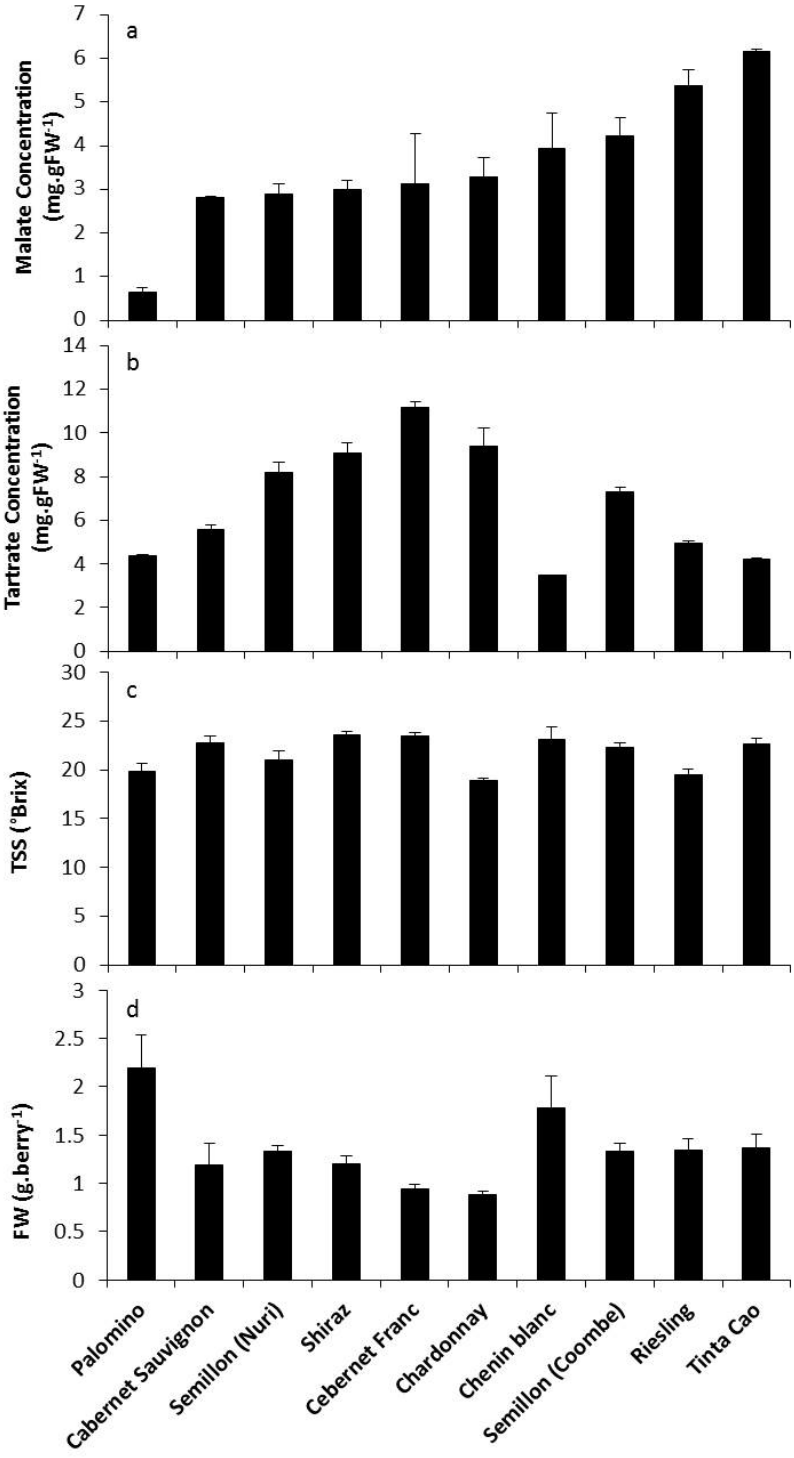


Figure 15: Berry fresh weight and composition across a range of cultivars at harvest from the 2010-11 season. Cultivars were plotted in order of increasing malate concentration for (a) malate concentration, (b) tartrate concentration, (c) TSS and (d) berry fresh weight at the time of harvest. Berries were collected from the Coombe vineyard at The University of Adelaide, Waite campus (Palomino, Cabernet Sauvignon, Chenin Blanc, Semillon, Riesling and Tinta Cao, where n = 4 ± SEM for all measurements except TSS, where n ≥ 12 ± SEM) or at the Nuriootpa Research Station vineyard (Semillon, Shiraz, Cabernet Franc and Chardonnay, where n = 3 ± SEM for all measurements except TSS, where n ≥ 12 ± SEM).

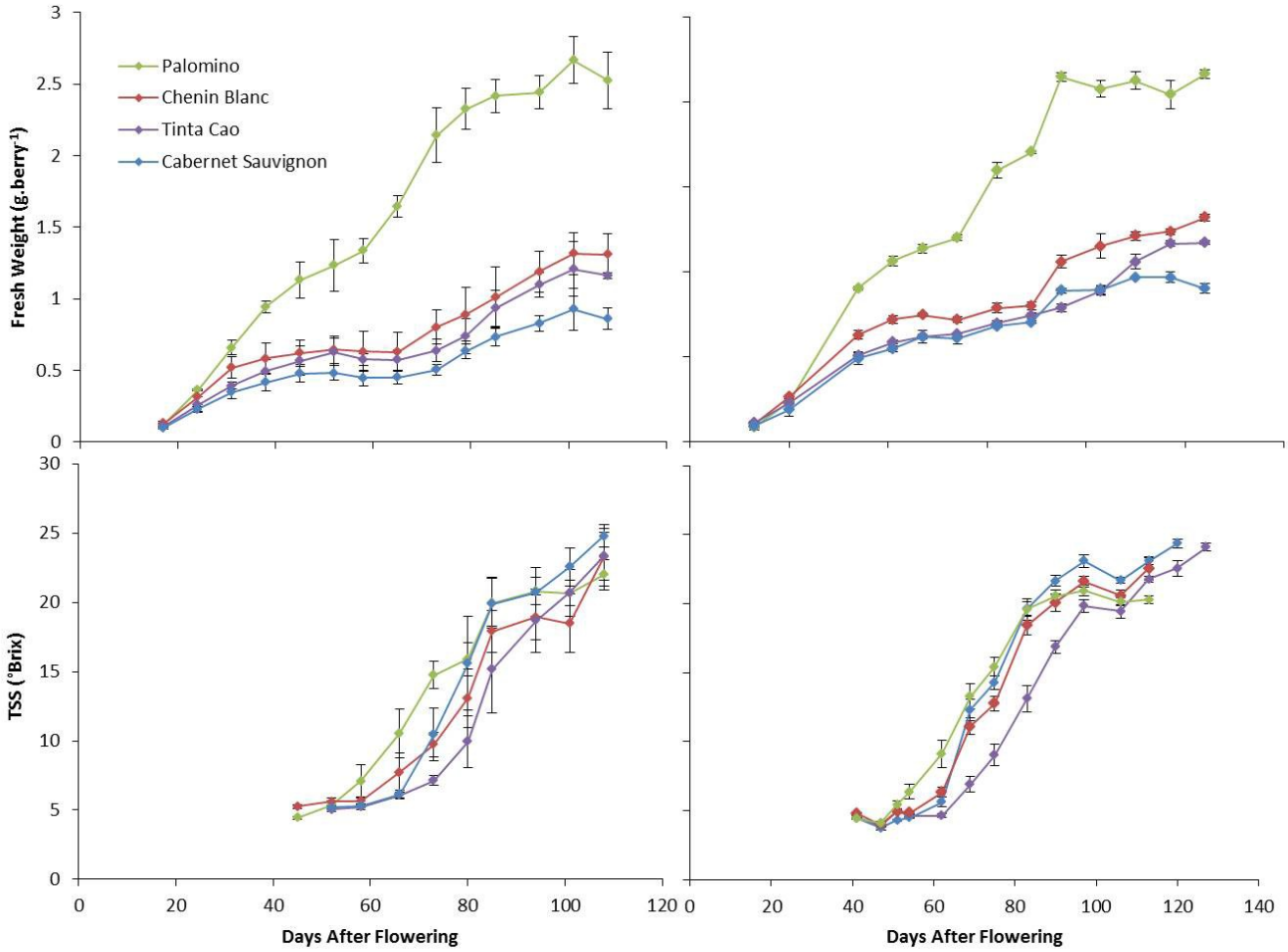


Figure 16: Fresh weight and TSS during development and ripening of four cultivars across two seasons. Season 2011-12 data (left) and season 2013-14 data (right) for fresh weight (top) and TSS (bottom). (n = 4 ± S.E.M.)

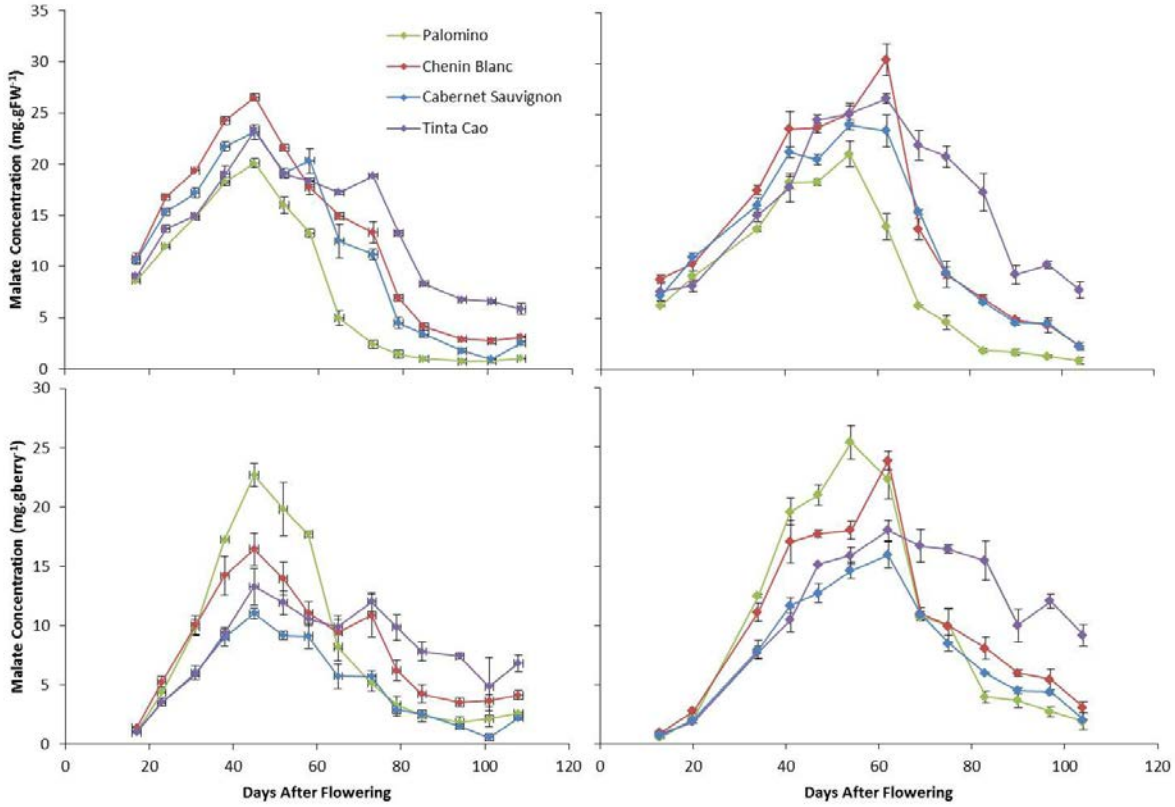


Figure 17: Malic acid levels during development and ripening of four cultivars across two seasons. Season 2011-12 data (left) and season 2013-14 data (right), presented as "mg per gram" (top) and "mg per berry" (bottom). (n = 4 ± S.E.M.)

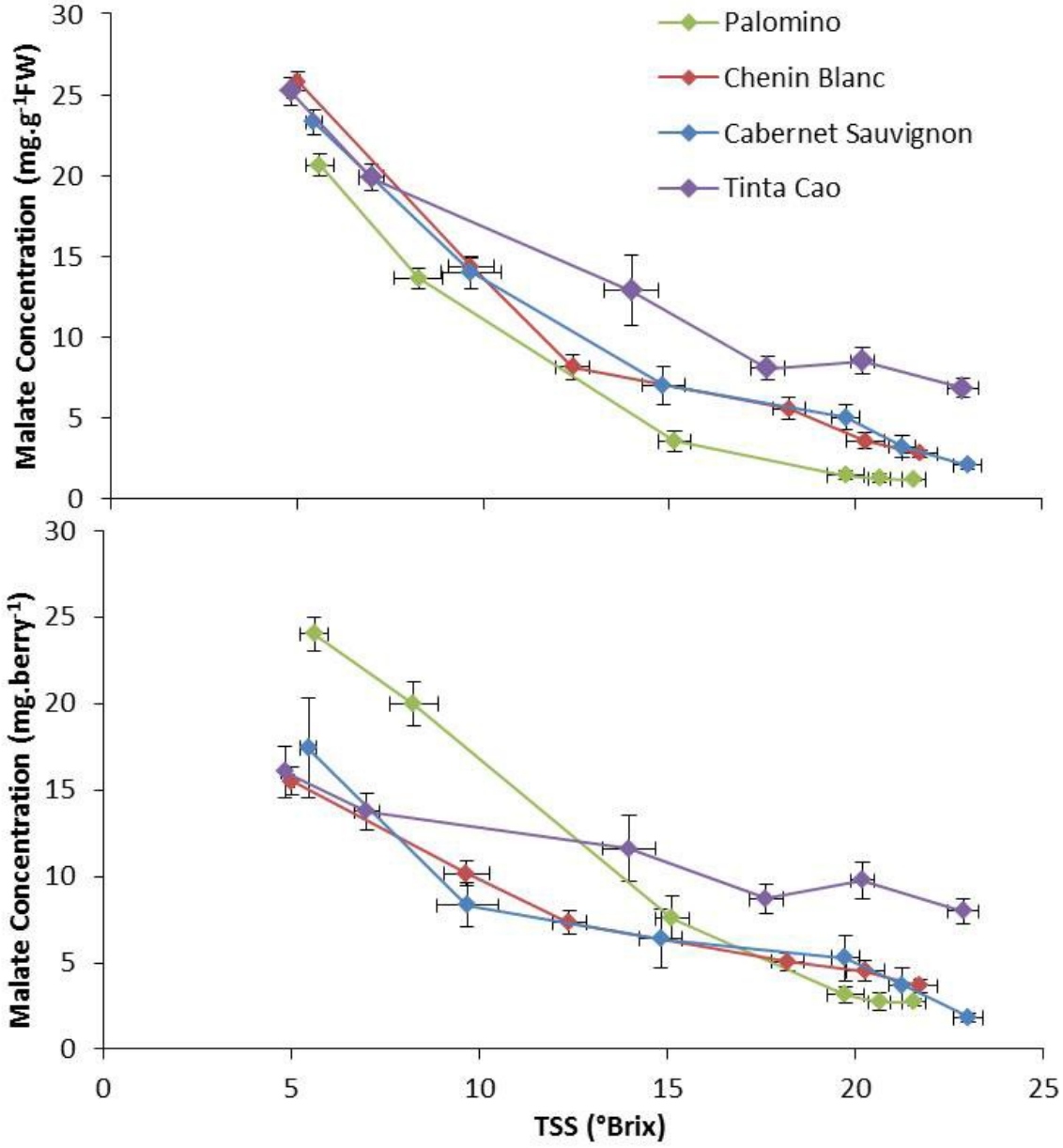


Figure 18: Malic acid levels of four cultivars pooled from two seasons and plotted against TSS. Presented as "mg per gram" (top) and "mg per berry" (bottom). Data are from the two seasons demonstrated individually in Figure 17 (i.e. 2011-12 and 2013-14). (n = 4 ± S.E.M.)

Table 1: Malic acid concentrations and losses (mg/gFW) from pooled 2011-12 and 2013-14 seasons.
Data are presented as malic acid concentration ($mg.g^{-1}$ fresh weight), or as otherwise indicated within brackets. Rates of malate loss were calculated over six weeks, beginning at the peak in malic acid concentration. (n=6 ± S.E.M.)

	Peak accumulation	Final harvest	Total malate lost (% of peak level)	Rate of malate loss per day (per °Brix)
Palomino	20.6 (±0.65)	1.2 (±0.09)	19.5 (94% w/w)	0.48 (1.79)
Cabernet Sauvignon	23.3 (±0.78)	2.1 (±0.24)	21.3 (91% w/w)	0.46 (1.74)
Chenin Blanc	25.8 (±0.58)	2.8 (±0.25)	23 (89% w/w)	0.51 (1.54)
Tinta Cao	25.2 (±0.87)	6.8 (±0.61)	18.4 (73% w/w)	0.31 (1.35)

Palomino, the lowest malic acid cultivar, produced berries that were significantly larger than the other three cultivars. However, levels of malic acid in Palomino berries were low whether calculated 'per gram' or 'per berry' and thus not due simply to dilution. Malic acid levels during development of the four cultivars showed similar patterns across the two seasons, therefore the data from both seasons were pooled and plotted against TSS, which was used to align berries from the different seasons based on developmental stage rather than calendar age. Upon pooling, the significant differences in malic acid levels between cultivars were retained, thus indicating that there was an inherent difference in the malic acid levels of the cultivars irrespective of seasonal conditions. However, a two-way ANOVA demonstrated that both cultivar ($p = 6.5E-12$) and season ($p = 0.0048$) led to differences in malic acid concentration, with cultivar explaining 87.5% of variability in the dataset. There was a significant level of interaction between cultivar and season ($p = 0.0002$). From these data it can be seen that genetic background had more of an influence on malate content than season, and that season affected malic acid concentrations of different cultivars in different ways.

Chenin Blanc lost the most malic acid during ripening (23 mg/gFW). However, because this cultivar accumulated a high concentration of the acid during Stage I development (25.8 mg/gFW), by harvest these berries still contained more malic acid than Palomino berries, which accumulated relatively low levels of malic acid (20.6 mg/gFW at the peak) and lost almost all of it by harvest. Both cultivars showed similar rates of malic acid loss, when expressed per day (Table 1) and per °Brix. Therefore the differences in malic acid concentration between these two white cultivars may be due to different synthesis and/or storage capabilities during development rather than degradation.

Cabernet Sauvignon and Tinta Cao accumulated similar levels of malic acid during Stage I (23-25 mg/gFW at the peak), while the rate of malic acid degradation in Tinta Cao was low compared to that of Cabernet Sauvignon, leading to more than a three-fold difference in malic acid concentration at harvest between the two cultivars. Therefore the difference between these two red cultivars may be in the ability to retain the acid in the vacuole during ripening, or due to different rates of malic acid degradation upon its release.

Overall these cultivars provide a useful basis for comparing metabolic pathways of malic acid at different stages of development. The white cultivars may be expected to show differences during berry development while the red varieties may be expected to show differences during ripening. Tinta Cao was of particular interest due to the high levels of malic acid present in the berries, and the low rates of malic acid loss during ripening.

Differences in malic acid between the four cultivars at harvest were significant, but small when compared to differences during development. However, the differences at harvest can have a large impact on the organoleptic characteristics of the berries. The threshold for sensory perception of malic acid is approximately 0.5 meq/L, or 70 mg/L (Blanco Gomis, 2000), therefore the difference between Palomino and Chenin Blanc (approximately 1.5 g/L malic acid, assuming 0.8 mL juice per gram of berry fresh weight), was approximately 20 times higher than the taste threshold. The difference between Tinta Cao and Palomino was even greater.

During the developmental seasons (i.e. from anthesis to the final harvest dates), season 2013-14 had a higher sum of degree days (2035.7°C) relative to Season 2011-12 (1963.8°C). This was also demonstrated in the mean monthly maximum temperature plot between the two seasons, whereby January, February and March mean maximum monthly temperatures were higher in 2013-14 than in 2011-12 (Figure 19). For these months the diurnal temperature ranges, suggested to influence malate metabolism (Sweetman et al. 2014), were also greater during the 2013-14 season.

As 2013-14 was a warmer season with larger diurnal temperature ranges during the ripening months, it would be expected that the fruit from this season would have lower final concentrations of malate. This was true for Cabernet Sauvignon and Chenin Blanc, although the differences were not significant. For Tinta Cao, there was a significantly higher malic acid concentration in berries harvested in 2013-14, which was unexpected due to the warmer conditions of that season. However, that season also saw a higher peak concentration of malic acid in Tinta Cao. When comparing the malic acid curves over time from both seasons (Figure 17), it can be seen that the warmer season demonstrated a longer acid-accumulation phase but condensed degradation phase, with steeper curves for malic acid loss in all cultivars except Tinta Cao. Overall, it appeared that malic acid levels in Tinta Cao berries were more resilient to higher temperatures than other cultivars tested.

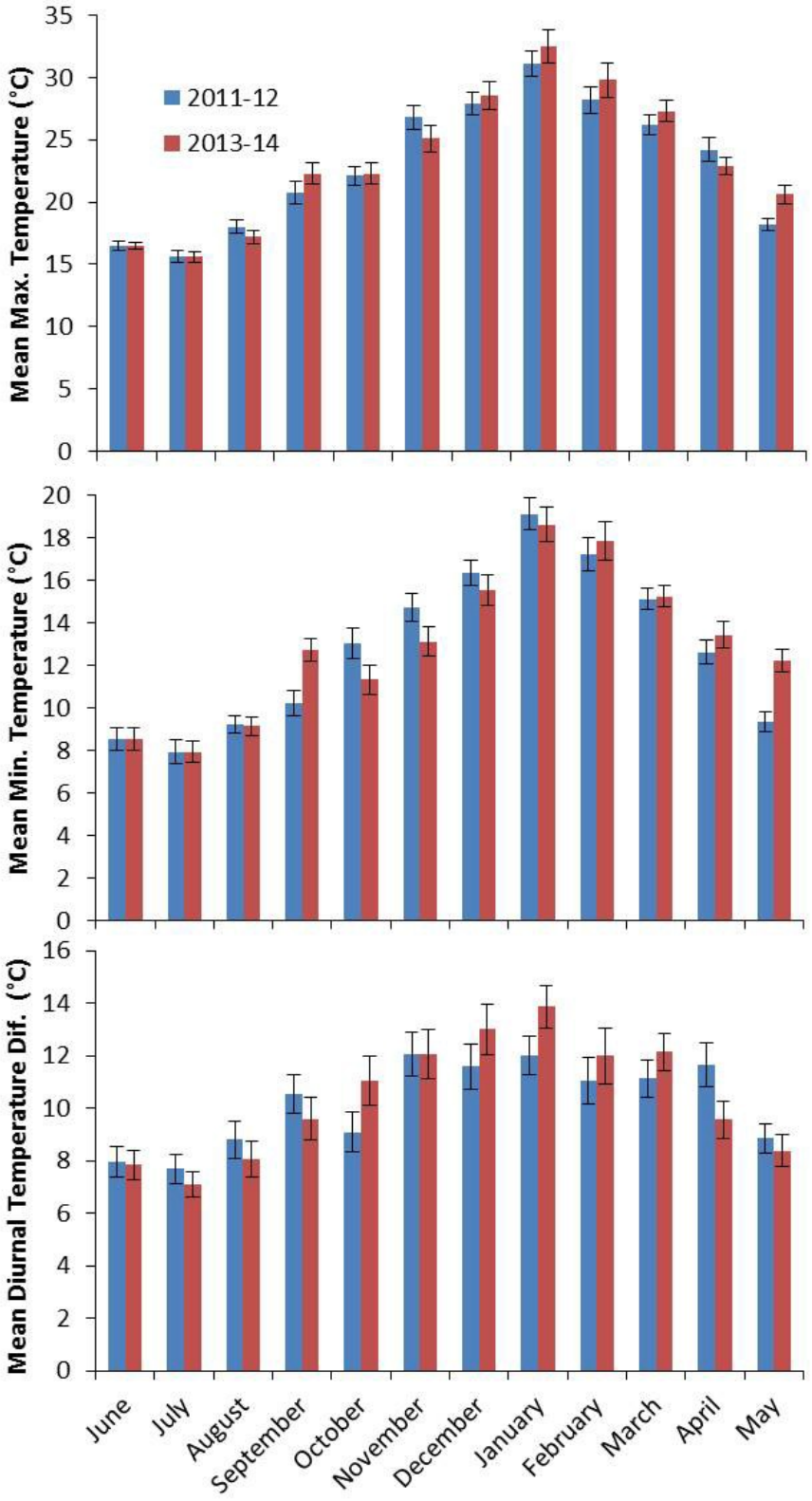


Figure 19: Monthly temperature means between the 2011-12 and 2013-14 seasons. Data were downloaded from the Bureau of Meteorology Kent Town meteorological station using the online Daily Weather Observations page (http://www.bom.gov.au/climate/dwo/IDCJDW0501.shtml).

6.3.2.1. Transcript Levels

To investigate further the potential causes of these differences in malic acid accumulation and degradation patterns, some gene transcript levels were measured using quantitative real-time reverse-transcriptase PCR and metabolite profiles were analysed. The aim of this experiment was to determine whether the differences in the patterns of malate accumulation during development, and degradation during ripening, could be linked to specific metabolic pathways, and whether these pathways could be aligned with those obtained from elevated temperature studies in Output 2.

The most obvious differences in malic acid concentration between cultivars occurred at véraison, by which time Palomino berries had already lost almost all malic acid, Chenin Blanc and Cabernet Sauvignon contained moderate levels, and Tinta Cao had high levels of malic acid. During this time the rate of malate loss in Tinta Cao was approximately half that of the other three cultivars. Therefore, this time-point was deemed important for exploring levels of other metabolites between Tinta Cao and the other three cultivars; however, samples from young, pre-véraison and ripening berries were also included in the analysis of transcript and metabolite levels.

Tinta Cao berries, which demonstrated low rates of malic acid loss during ripening, also demonstrated high transcript levels of the mitochondrial malic enzyme gene, *VvNadme1* (Figure 20) and the cytosolic malate dehydrogenase gene, *VvCmdh1* (Figure 21) during véraison and/or ripening. This was surprising, considering that these genes were also up-regulated in response to elevated temperature in Shiraz (Output 2), and was therefore thought to be related to increased malate catabolism. However, Tinta Cao berries also showed high *VvPepc1* and *VvPepc3* transcript levels (Figure 22) as well as elevated *VvPpase* and *VvAmlt9* transcript levels (Figure 23) relative to the other cultivars investigated in this study. Together, these data suggest that the lower rates of malate loss during ripening in Tinta Cao berries may be due to prolonged malate sequestration in the vacuole and enhanced malic acid regeneration during ripening, rather than a decrease in the availability of malic acid catabolic enzymes. As there may be some recycling of malic acid during ripening, the high level of *VvPepck* transcript also observed during véraison and ripening of these berries (Figure 24) could also represent enhanced PEP biosynthesis from malic acid (via oxaloacetate) in these berries, although this was unlikely to support gluconeogenesis, as sugar levels were typically low in this cultivar (see "Metabolite Levels", below). Interestingly, Tinta Cao berries showed strikingly low transcript levels of the Heat Shock protein gene, *VvHsp18.2* (Figure 26). This may be due to the large canopy size of Tinta Cao, which could help to protect the berries against adverse environmental conditions such as high light exposure and heat. This likely also contributed to the increased levels of malic acid observed in the fruit at harvest, although genes that were typically up-regulated in response to elevated

temperature treatments in Shiraz (Output 2), such as *VvNadme1* and *VvCmdh1* (Figure 6 and 7), were also elevated in Tinta Cao.

Cabernet Sauvignon berries, which showed similar malic acid accumulation to Tinta Cao, but large losses of malic acid during ripening, demonstrated lower transcript levels of *VvNadpme1*, *VvNadme1* and *VvNadme2* during ripening (Figure 20), as well as lower transcript levels of *VvCmdh1* and *VvCmdh3* (Figure 21) and all three *VvPepc* genes (Figure 23), relative to Tinta Cao berries. These berries also demonstrated lower transcript levels of the *VvAlmt9* transporter during ripening, and lower transcript levels of some of the *VvPpase* genes during véraison (Figure 23). Therefore this cultivar seemed to be less equipped for malic acid sequestration in the vacuole and malic acid regeneration during ripening, when compared to Tinta Cao berries.

Chenin Blanc berries, which showed high accumulation of malic acid during development and high rates of malic acid loss during ripening, demonstrated high pre-véraison transcript levels of two NADP-dependent malic enzyme genes, *VvNadpme1* and *VvNadpme2* (Figure 20) and low levels of cytosolic and mitochondrial malate dehydrogenase gene transcripts *VvCmdh1*, *VvCmdh2* and *VvMmdh1* (Figure 21). During véraison and ripening these patterns generally switched, to lower transcript levels of *VvNadme1* and *VvNadme2* (Figure 20) and higher transcript levels of *VvCmdh1* and *VvCmdh3* (Figure 21). This cultivar also showed typically low levels of PEP carboxylase gene transcript levels throughout development and ripening (Figure 22), despite showing high accumulation of malic acid until véraison. Therefore, malic acid synthesis in this cultivar could be supported by the conversion of pyruvate to malate through the activity of the highly-expressed malic enzymes, rather than the conversion of PEP to malate through the PEP carboxylase and malate dehydrogenase route. Chenin Blanc berries showed generally higher transcript levels of the vacuolar pyrophosphatase genes during early development, pre-véraison and even véraison stages (Figure 23), which may have facilitated the high accumulation of malic acid during development. However, the high *VvTdt* transcript levels and low *VvAlmt9* transcript levels at véraison may have limited the potential for retention of malic acid in the vacuole during ripening, thus resulting in low levels of the acid at harvest.

Palomino showed low accumulation of malic acid during development and moderate losses during ripening. This cultivar typically demonstrated moderate levels of malic enzyme, malate dehydrogenase and PEP carboxylase transcripts during early development, and high levels of *VvCmdh1*, *VvCmdh2* and *VvMmdh1* transcripts (Figure 21) accompanied by low *VvPepc2* and *VvPepc3* transcripts (Figure 22) at véraison. This cultivar also showed generally low transcript levels of all *VvPpase* genes, particularly at véraison but also pre-véraison, as well as high levels of the *VvTdt* transcript particularly during ripening (Figure 23). Therefore, this cultivar was probably incapable of storing high concentrations of malic acid within the vacuole, thus rendering the acid vulnerable to metabolic activities of the

enhanced malate dehydrogenases, and with low capacity for malic acid regeneration due to low PEP carboxylase transcript during ripening.

With respect to tartaric acid, Chenin Blanc berries demonstrated the lowest levels at harvest, of all cultivars studied in this, and all other experiments (Figure 15), and generally demonstrated the lowest level of L-idonate dehydrogenase gene transcript of the four cultivars studied within this experiment (Figure 25). Tartaric acid was highest in Cabernet Sauvignon berries at véraison and during ripening. Transcript levels of L-Idh1 at véraison were highest in Cabernet Sauvignon and Palomino berries. High L-Idh1 expression could enable continued biosynthesis of tartaric acid during ripening, thus explaining the relatively high tartaric acid concentration in Cabernet Sauvignon berries. However, Palomino berries, which also demonstrate high L-Idh1 transcript level at véraison, had a low tartaric acid concentration, due to a large loss during development. This large loss of tartaric acid in Palomino berries between young and ripe berries is likely due to the dramatic increase in berry size during this time, causing a high dilution effect. The L-idonate dehydrogenase enzyme is the only confirmed enzyme of the tartaric acid biosynthetic pathway (DeBolt *et al.*, 2006), and these data suggest that the expression of this protein could regulate tartaric acid accumulation in the berry and may therefore represent a rate-limiting step in the biosynthetic pathway.

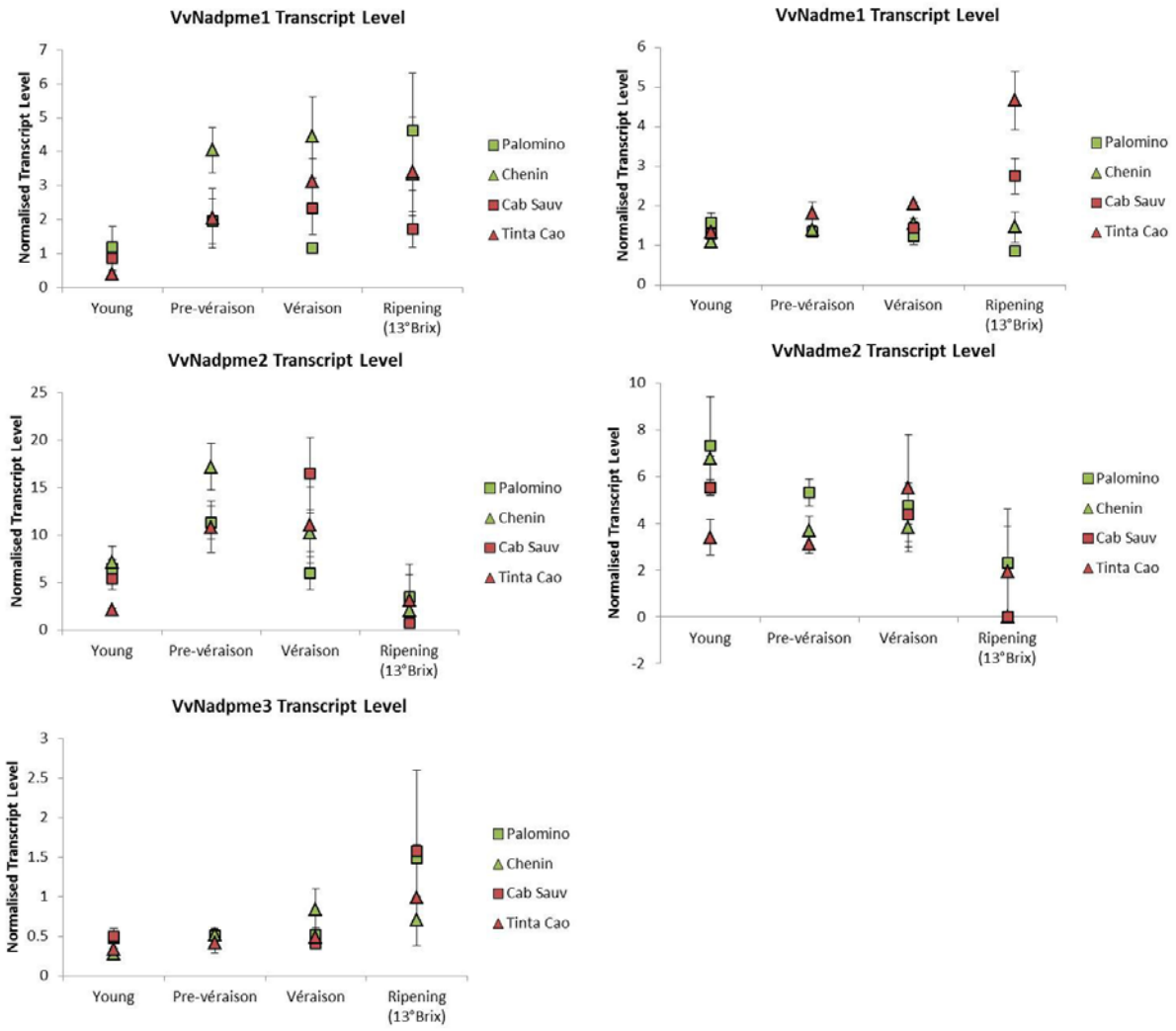


Figure 20: Transcript levels of malic enzyme genes between the four cultivars. Cytosolic/chloroplastic (NADP-dependent) ME genes are presented on the left, mitochondrial (NAD- dependent) ME genes are presented on the right. Samples were analysed from young berries (two weeks before véraison), pre-véraison (one week before véraison), during véraison (based on skin colour change or transparency) and during ripening (at approximately 13°Brix). (n = 3 ± S.E.M.)

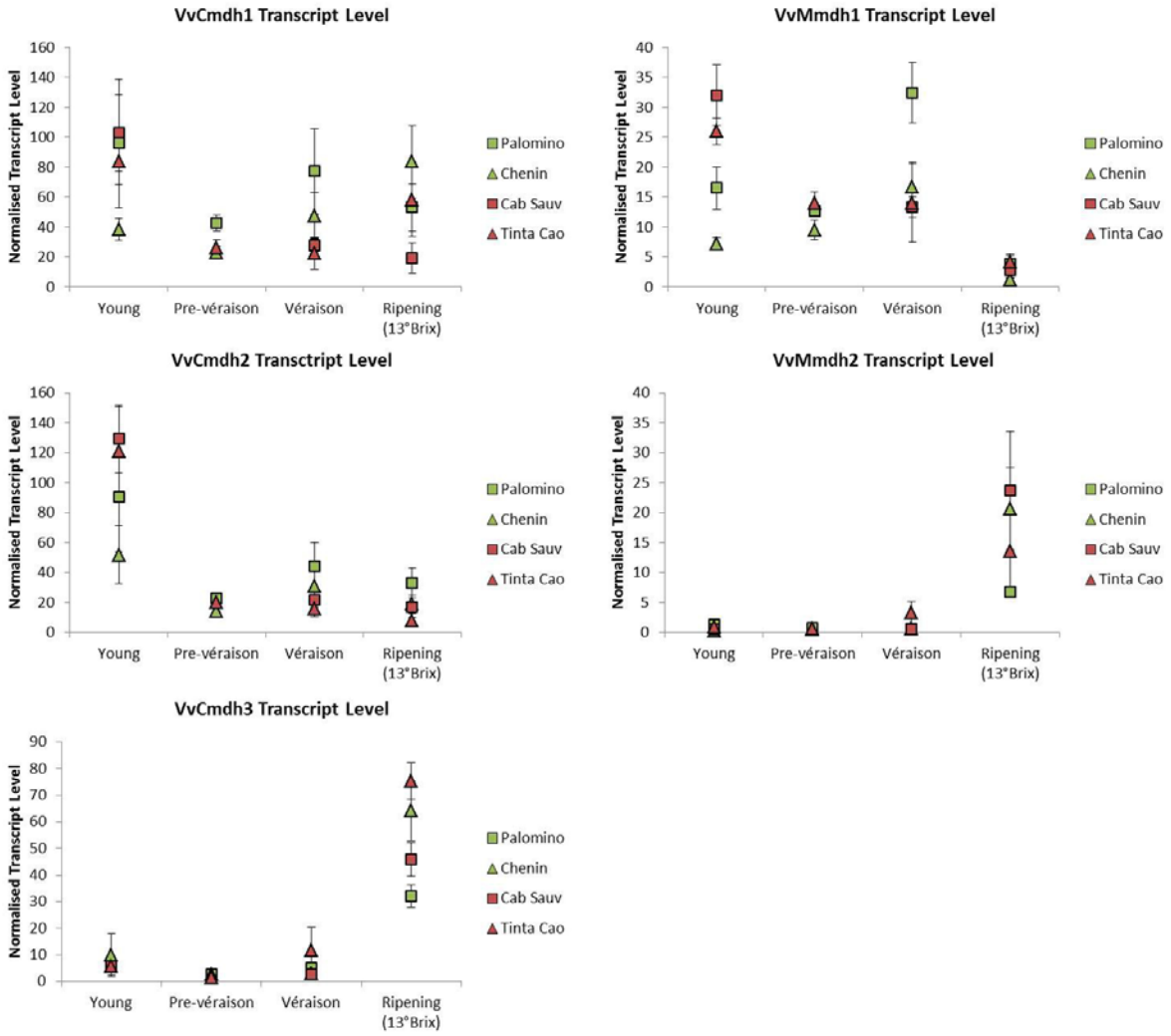


Figure 21: Transcript levels of malate dehydrogenase genes between the four cultivars. Cytosolic MDH genes are presented on the left, mitochondrial MDH genes are presented on the right. Samples were analysed from young berries (two weeks before véraison), pre-véraison (one week before véraison), during véraison (based on skin colour change or transparency) and during ripening (at approximately 13°Brix). (n = 3 ± S.E.M.)

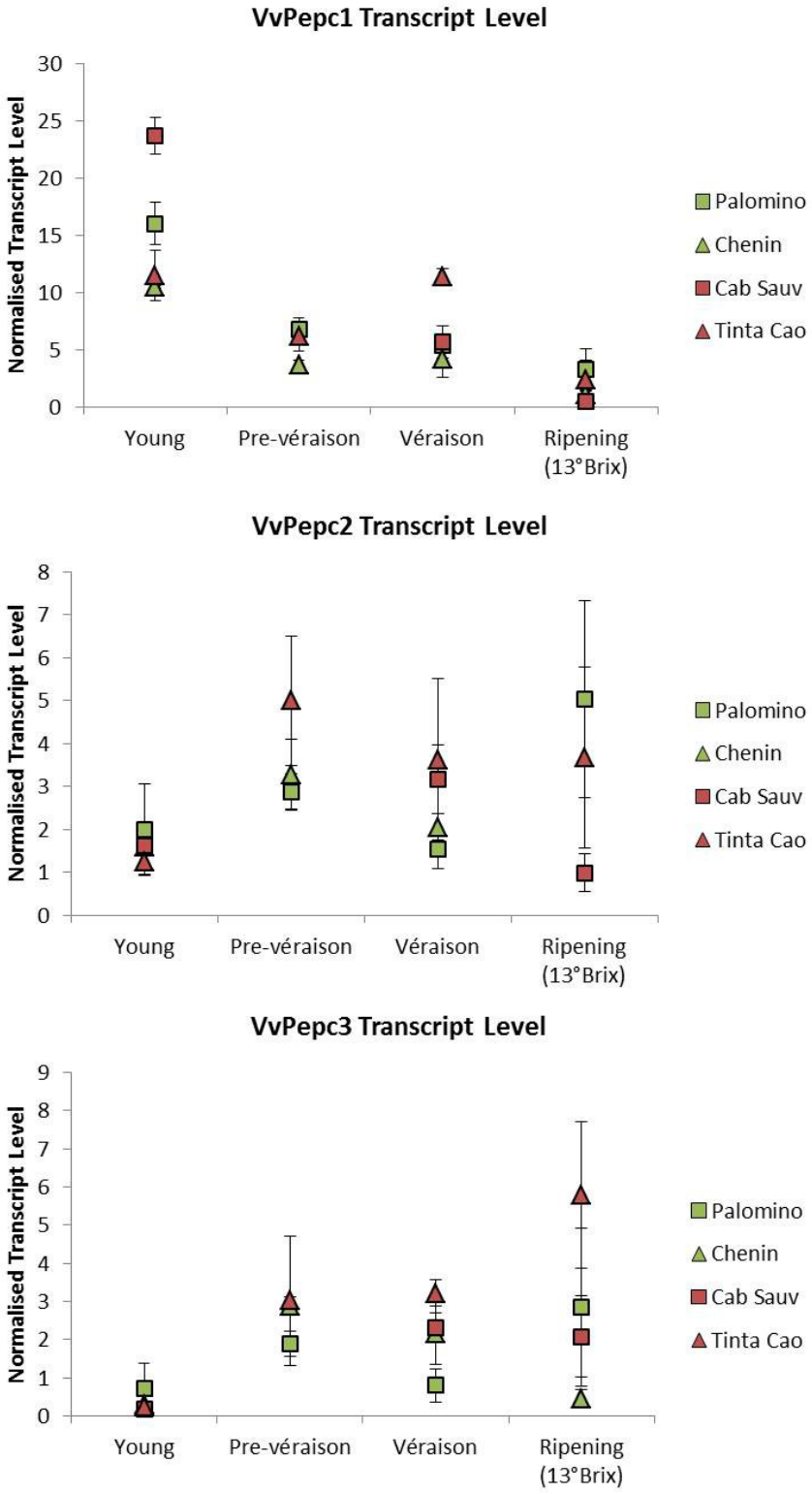


Figure 22: Transcript levels of phosphoenolpyruvate carboxylase genes between the four cultivars. Samples were analysed from young berries (two weeks before véraison), pre-véraison (one week before véraison), during véraison (based on skin colour change or transparency) and during ripening (at approximately 13°Brix). (n = 3 ± S.E.M.)

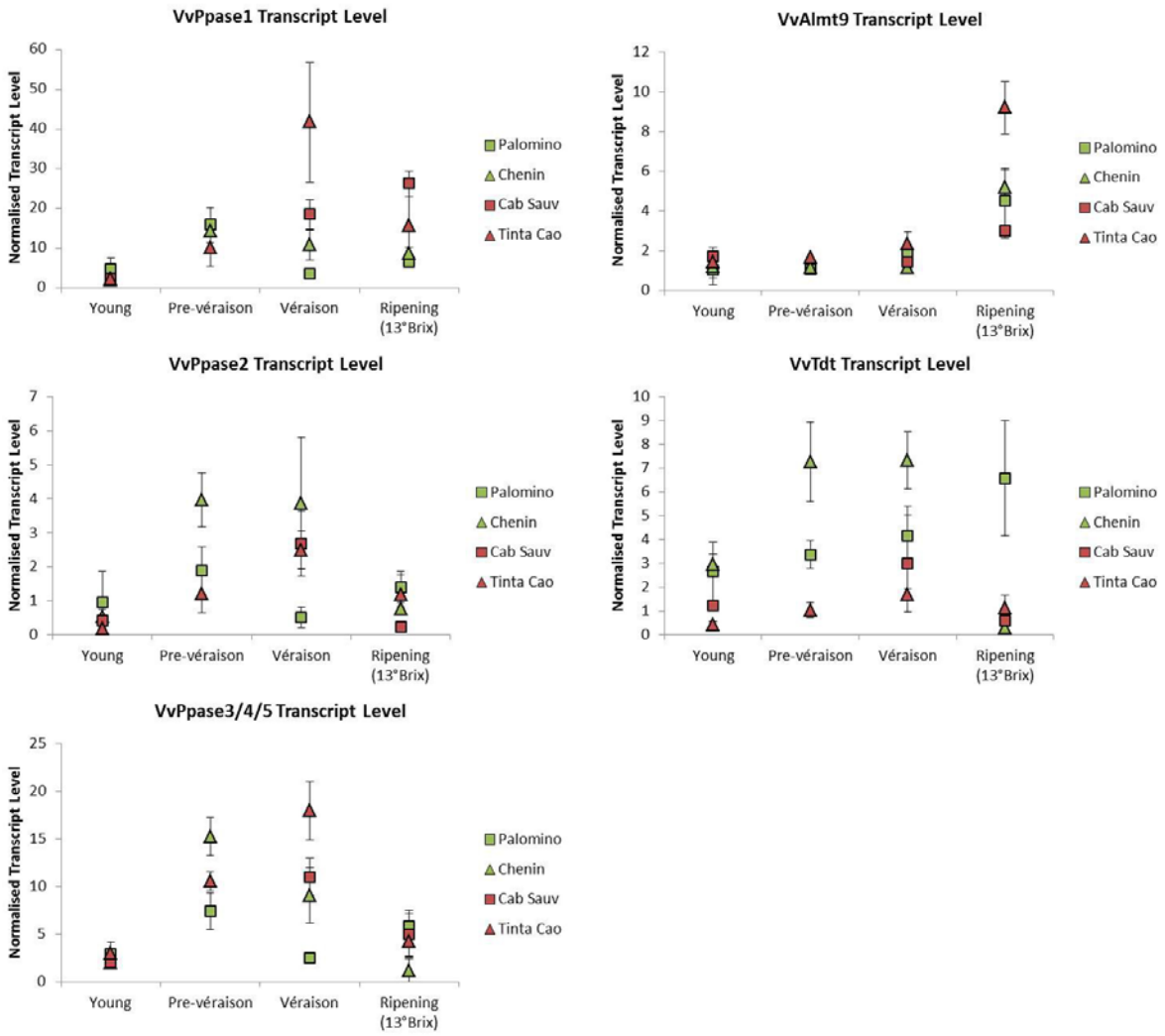


Figure 23: Transcript levels of vacuolar transporter genes between the four cultivars. Vacuolar Pyrophosphatase genes are presented on the left, malic acid transporter genes are presented on the right. *VvVppase3/4/5* refers to genes that could not be assayed individually due to high sequence identity at the nucleotide level. Samples were analysed from young berries (two weeks before véraison), pre-véraison (one week before véraison), during véraison (based on skin colour change or transparency) and during ripening (at approximately 13°Brix). (n = 3 ± S.E.M.)

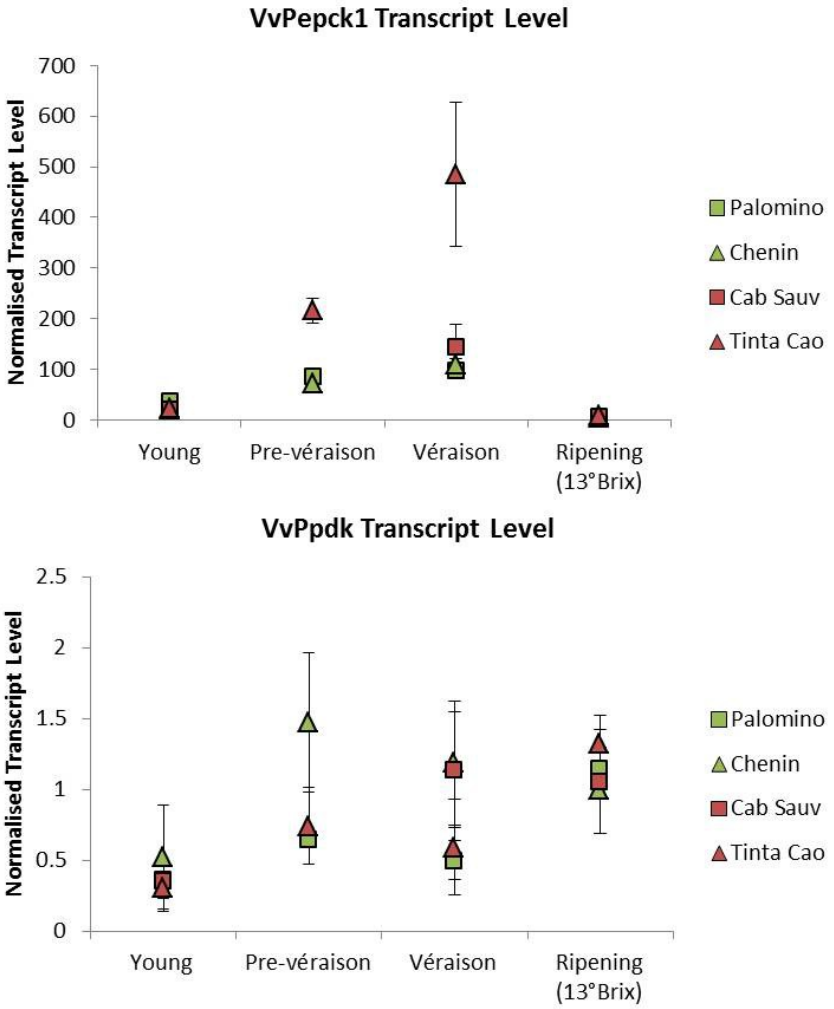


Figure 24: Transcript levels of genes involved in gluconeogenesis between the four cultivars. Samples were analysed from young berries (two weeks before véraison), pre-véraison (one week before véraison), during véraison (based on skin colour change or transparency) and during ripening (at approximately 13°Brix). (n = 3 ± S.E.M.)

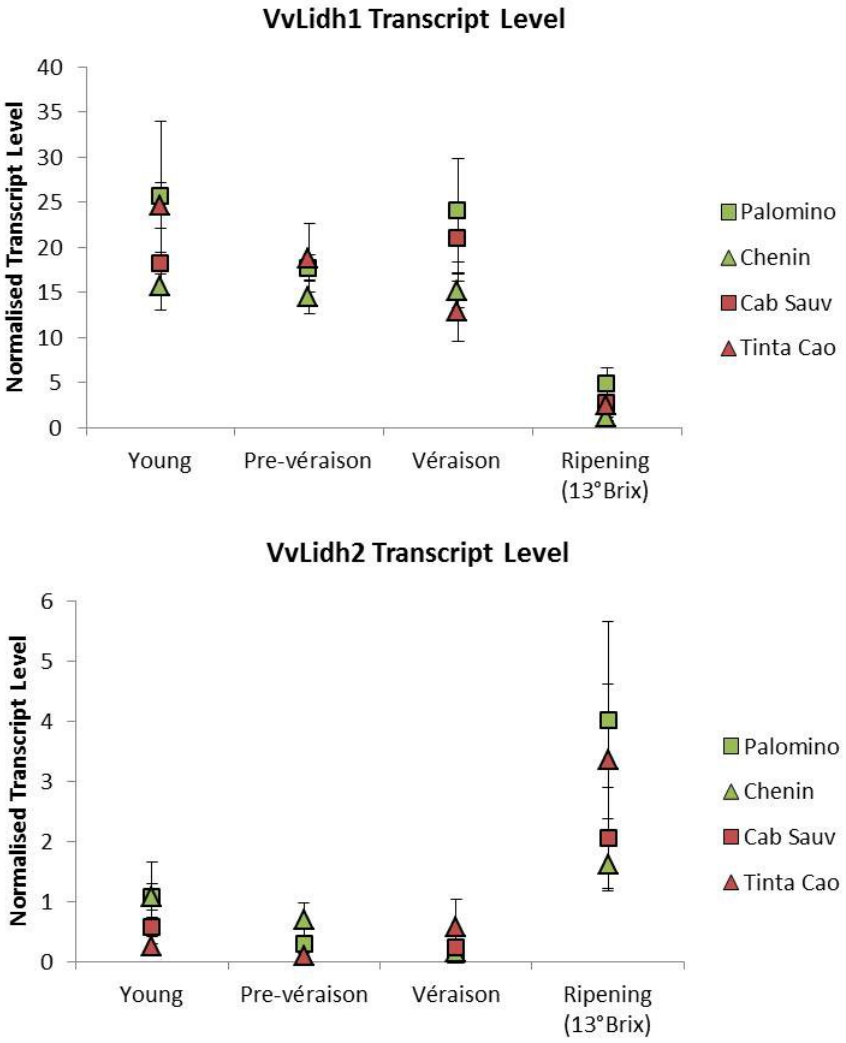


Figure 25: Transcript levels of L-idonate dehydrogenase genes between the four cultivars. Samples were analysed from young berries (two weeks before véraison), pre-véraison (one week before véraison), during véraison (based on skin colour change or transparency) and during ripening (at approximately 13°Brix). (n = 3 ± S.E.M.)

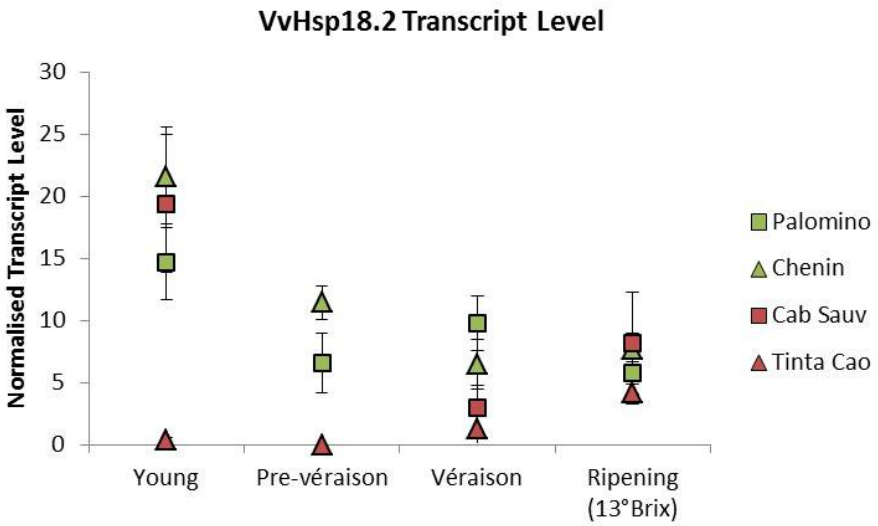


Figure 26: Transcript levels of heat-shock protein gene, *VvHsp18.2* between the four cultivars. Samples were analysed from young berries (two weeks before véraison), pre-véraison (one week before véraison), during véraison (based on skin colour change or transparency) and during ripening (at approximately 13°Brix). (n = 3 ± S.E.M.)

6.3.2.2. Metabolite Levels:

A metabolic profile of the four cultivars was conducted using GC/MS, and the findings are summarised in Figures 27-30 (cultivars represented by individual figures). Patterns of malic acid levels in the four cultivars as measured by GC/MS were consistent with the more detailed patterns measured throughout development using HPLC. In general, during véraison and ripening Tinta Cao berries, which retained high levels of malic acid, had low levels of most other metabolites, particularly organic acids (tartaric, succinic, gluconic, quinic and glucaric/galactaric acids) and amino acids (phenylalanine, threonine, B-alanine, valine, leucine, isoleucine, serine, aspartic acid, glutamic acid, proline and GABA). The levels of sugars (fructose, glucose and sucrose) were also lower, as were putrescine, glycerol and caffeic acid (Figure 27). This suggests that at véraison and ripening, Tinta Cao berries have either enhanced catabolism or reduced synthesis of amino acids and sugars relative to the other cultivars. When taken together with the findings from Output 2, whereby elevated temperature led to a decrease in malic acid and a general increase in amino acid levels, it seems that malic acid levels in berries may be negatively correlated with amino acid levels, and that the balance between organic acid and amino acid metabolism can be altered by genotype and environmental conditions. It may be that altered malic acid availability influences amino acid metabolism (i.e. if insufficient malic acid is available, then amino acids are degraded to make available carbon skeletons for ripening), or that altered amino acid metabolism influences malic acid levels (i.e. high rates of amino acid degradation lead to decreased requirements for malic acid, and thus higher retention of the acid in the vacuole). Factors that regulate carbon and nitrogen balance in the berry cell are thus expected to strongly influence malic acid concentration in grape berries. In most cases amino acid levels decreased throughout véraison and ripening and therefore indeed could be catabolised to provide carbon skeletons for ripening under normal circumstances. Furthermore, in young berries the amino acids valine, leucine, isoleucine, as well as the amine ethanolamine were much higher in Tinta Cao relative to the other cultivars, and dropped by véraison to levels as low as, or even lower than the other cultivars (Figure 27). These amino acids in particular could have been catabolised to support metabolism in ripening Tinta Cao berries.

Alternatively, if amino acids are synthesised during ripening, then the lower levels of most amino acids observed in Tinta Cao could be due to reduced availability of malic acid as a source of carbon backbones. In Tinta Cao berries, amino acids stemming from pyruvate metabolism (valine, leucine, B-alanine, serine) as well as oxaloacetate (aspartate, threonine) were lower than in other cultivars. Both pyruvate and oxaloacetate are readily supplied by malate-metabolising activities of malic enzyme and malate dehydrogenase, respectively; both of which were represented relatively highly at the

transcript level in Tinta Cao during véraison and/or ripening (*VvNadme1* and *VvCmdh1* genes specifically). However, it was suggested that the efflux of malic acid from the vacuole is retarded in Tinta Cao berries during véraison and ripening due to high transcript levels of *VvAlmt9* and *VvPpase* genes, which would impact on numerous avenues of metabolism simultaneously.

The lower levels of sugars observed during the ripening of these berries could suggest either impaired gluconeogenesis due to reduced availability of malic acid, or slower import of sugars from the leaves. A decrease in sugar supply could also explain the decrease in amino acid levels, but it does not explain the higher levels of malic acid, which, if sugars are in short supply, may be expected to be exported and used as an alternate carbon source, rather than retained at high levels in the vacuole. The high transcript level of *VvPepck* suggests that gluconeogenesis could be enhanced, if a suitable carbon source is available. However, as gluconeogenesis from malic acid can only account for a very low proportion of sugar accumulation, it is more likely that the lower sugar levels are a result of reduced sugar import from other parts of the vine. Unfortunately absolute levels of sugars were not quantified using the GC-MS method therefore a direct comparison of glucose, fructose and sucrose levels with malate levels cannot be given. Tinta Cao vines exhibited a large canopy and were therefore unlikely to be in short supply of carbohydrates, and further the levels of total soluble solids were as high as the other cultivars at the final harvest (~24°Brix) consistent with 24.3°Brix seen in mature Tinta Cao berries by (Kliewer *et al.*, 1967).

Citric acid levels were higher in Tinta Cao berries relative to the other cultivars. To look for correlations between malic acid and various other metabolites, scatterplots were generated for individual cultivars, using data from the ripening stages. The correlations between malic acid and citric acid levels (R^2 values of 0.71, 0.83 and 0.96 for Cabernet Sauvignon, Chenin Blanc and Palomino, respectively) were typically lower than the correlations between malic acid and succinic acid levels (R^2 = 0.85, 0.95 and 0.98, respectively). However, in the case of Tinta Cao, there was a significantly higher correlation between malic and citric acid (R^2 = 0.90) than malic and succinic acid (R^2 = 0.60). Therefore the metabolism of malic acid may be closely linked to citric acid in Tinta Cao berries, and a regulatory point for malic acid accumulation could therefore occur with any of the TCA cycle enzymes that exist between citric acid and succinic acid. For example, a decrease in aconitase activity, which converts citrate to isocitrate would be expected to cause an accumulation of citrate and would interrupt flux through the TCA cycle, potentially leading to a decrease in subsequent intermediates (e.g. succinic acid) and an accumulation of upstream intermediates (i.e. malic acid, an anaplerotic supply carbon for the TCA cycle during ripening). Recently, a study by Morgan et al. (2013) demonstrated, through a tomato introgression line, that tomato fruit exhibiting higher malic acid and citric acid levels and lower fumaric acids levels had reduced cytosolic aconitase activity. In

these fruit, malic acid levels were approximately 50% higher. This seems to correspond with the results seen for Tinta Cao grape berries, however in the case of the tomato fruit, amino acid levels were generally up-regulated rather than down-regulated. Isocitrate dehydrogenase, another TCA-cycle enzyme, could also be a candidate. Inhibition of either aconitase or isocitrate dehydrogenase would reduce flux to α-ketoglutarate. In Tinta Cao the levels of glutamate, GABA, proline and putrescine were much lower than in other cultivars, each of which stem from α-ketoglutarate metabolism. Activities of these TCA cycle enzymes as well as vacuolar transporters need to be determined in Tinta Cao berries and compared with other cultivars, to determine whether malic acid levels are more likely determined by sequestration in the vacuole, or functionality of the TCA cycle during ripening. The lower levels of glutamate, GABA, proline and putrescine could also indicate lower levels of abiotic stress, consistent with lower transcript levels of the Hsp18.2 heat shock protein gene seen in Tinta Cao berries (See "Transcript Levels"), and are potentially accounted for by reduced light exposure or cooler temperatures around the bunches as a result of the large canopy.

Cabernet Sauvignon accumulated high levels of stress-related metabolites during véraison and ripening, including GABA, proline and putrescine (Figure 28). As Proline and GABA are indicative of a stress response, and were seen to be significantly elevated in Shiraz berries exposed to high temperatures, it may be that Cabernet Sauvignon berries were exposed to a stress during véraison and ripening. The fruit of the Cabernet Sauvignon vines were highly exposed relative to Tinta Cao berries, which were very well shaded by the larger canopy of this cultivar, and therefore may have reached higher temperatures during the day. Cabernet Sauvignon berries also showed an increase in *VvHsp18.2* transcript level during véraison and ripening; a gene that was also shown to be responsive to heat stress (Output 2). Although the levels of this transcript were not significantly higher than in Palomino or Chenin Blanc berries.

Chenin Blanc berries contained moderate levels of malic acid. Transcript levels of genes encoding proteins thought to be involved in both malic acid import and export from the vacuole seemed to be high in this cultivar, suggesting a potentially high turnover of malic acid. High transcript levels of NADP-malic enzyme could mean that a large proportion of this malic acid is converted to or from pyruvate. As there are also high levels of serine and threonine (Figure 29) it may be that pyruvate, derived from malic acid as it released from the vacuole, is fed toward amino acid biosynthesis.

Palomino berries, which had low levels of malic acid, also had low levels of other TCA cycle intermediates (Figure 30). Due to the lower levels of citric acid and higher levels of oxalic acid in this cultivar, citrate may be diverted from the TCA cycle to the glyoxylate cycle and oxalic acid accumulation, thus decreasing flux through the TCA cycle. Notably, this cultivar demonstrated higher transcript levels of the tonoplast dicarboxylate transporter gene and lower levels of the *VvPpase* genes that

may both contribute to a decrease in the capacity for malic acid storage in the vacuole. Increased efflux of malic acid could lead to its rapid loss, potentially through cytosolic malate dehydrogenase, for which transcript levels were relatively high in this cultivar.

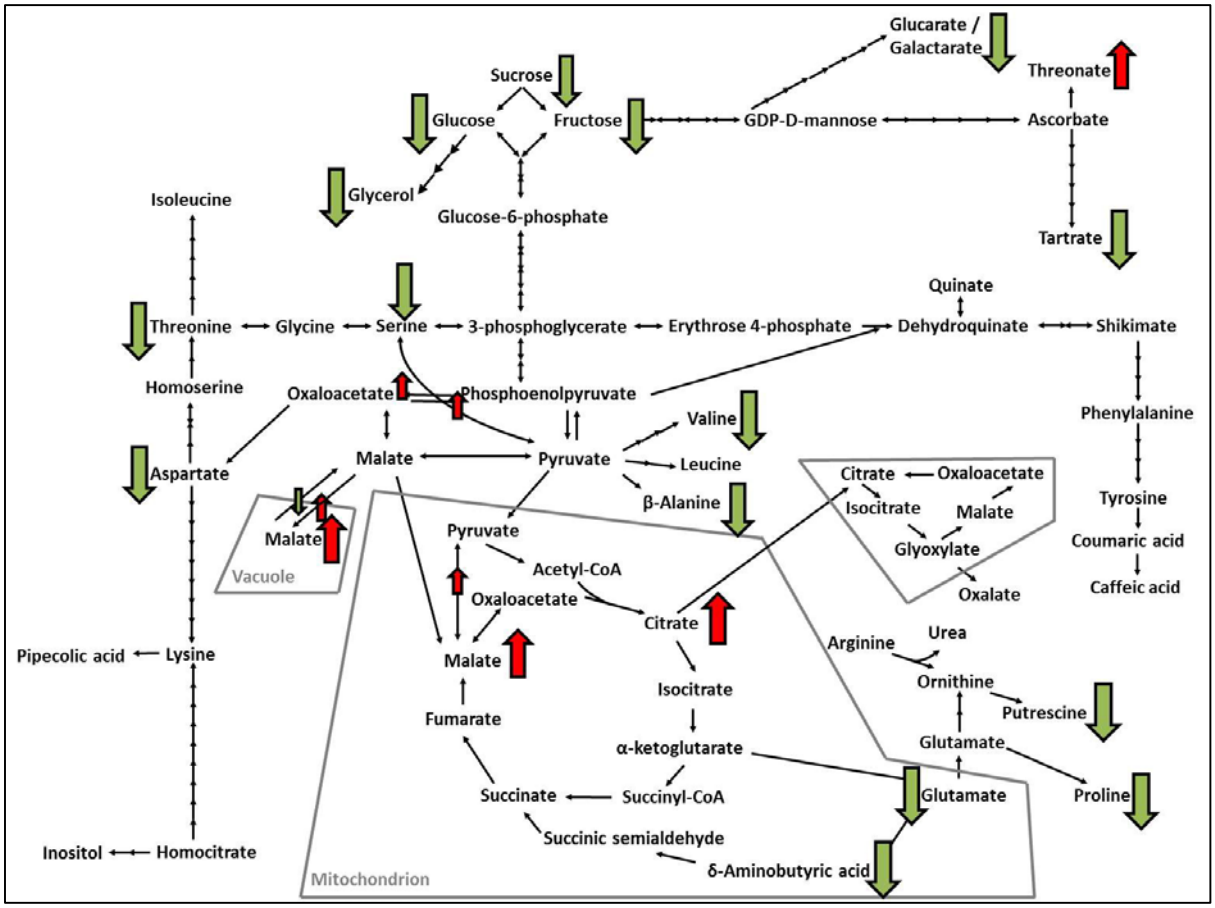


Figure 27: Tinta Cao ripening transcript and metabolite model. Large arrows represent increases (up-facing, red arrow) or decreases (down-facing, green arrow) in metabolite levels relative to other cultivars. Smaller arrows represent increases or decreases in transcript levels. (n = 4 ± S.E.M.).

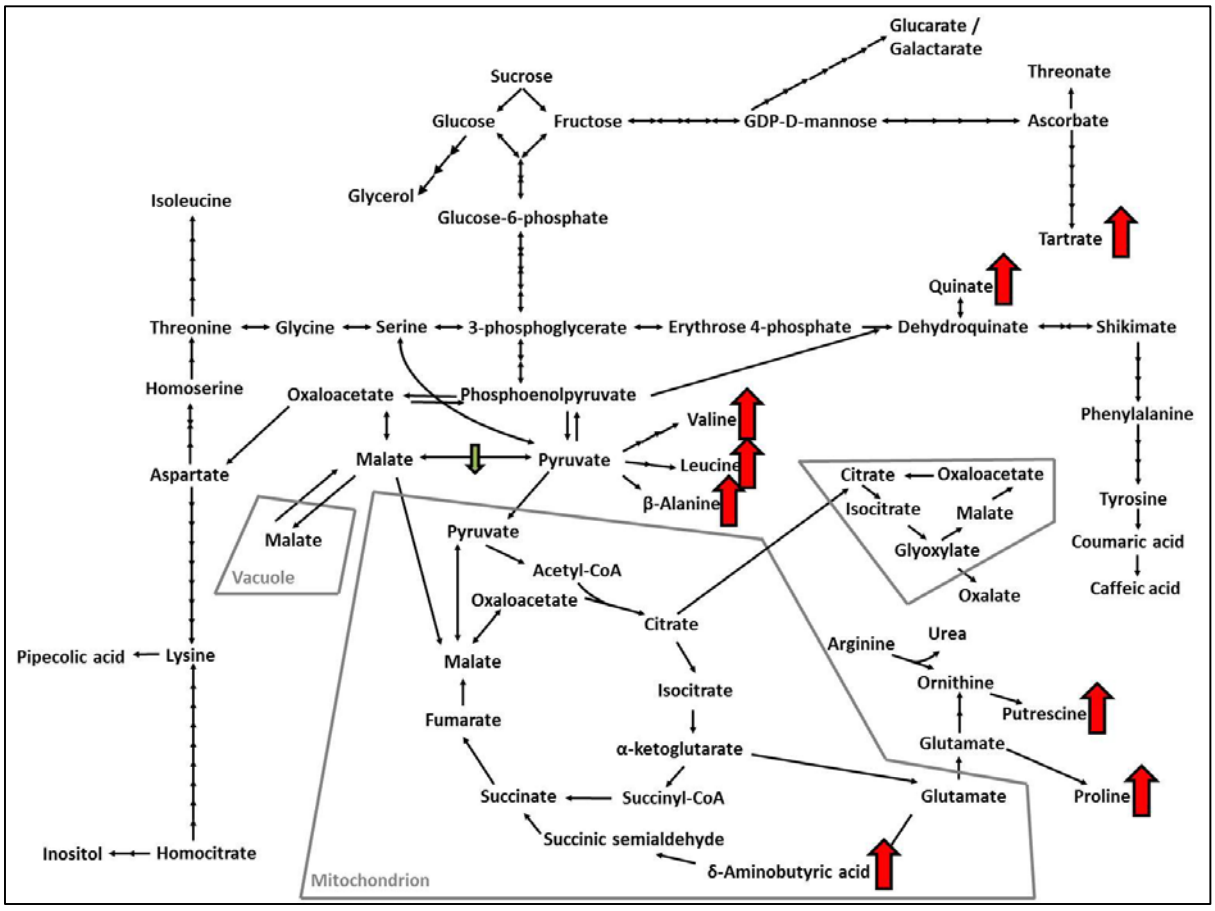


Figure 28: Cabernet Sauvignon ripening transcript and metabolite model. Large arrows represent increases (up-facing, red arrow) or decreases (down-facing, green arrow) in metabolite levels relative to other cultivars. Smaller arrows represent increases or decreases in transcript levels. (n = 4 ± S.E.M.).

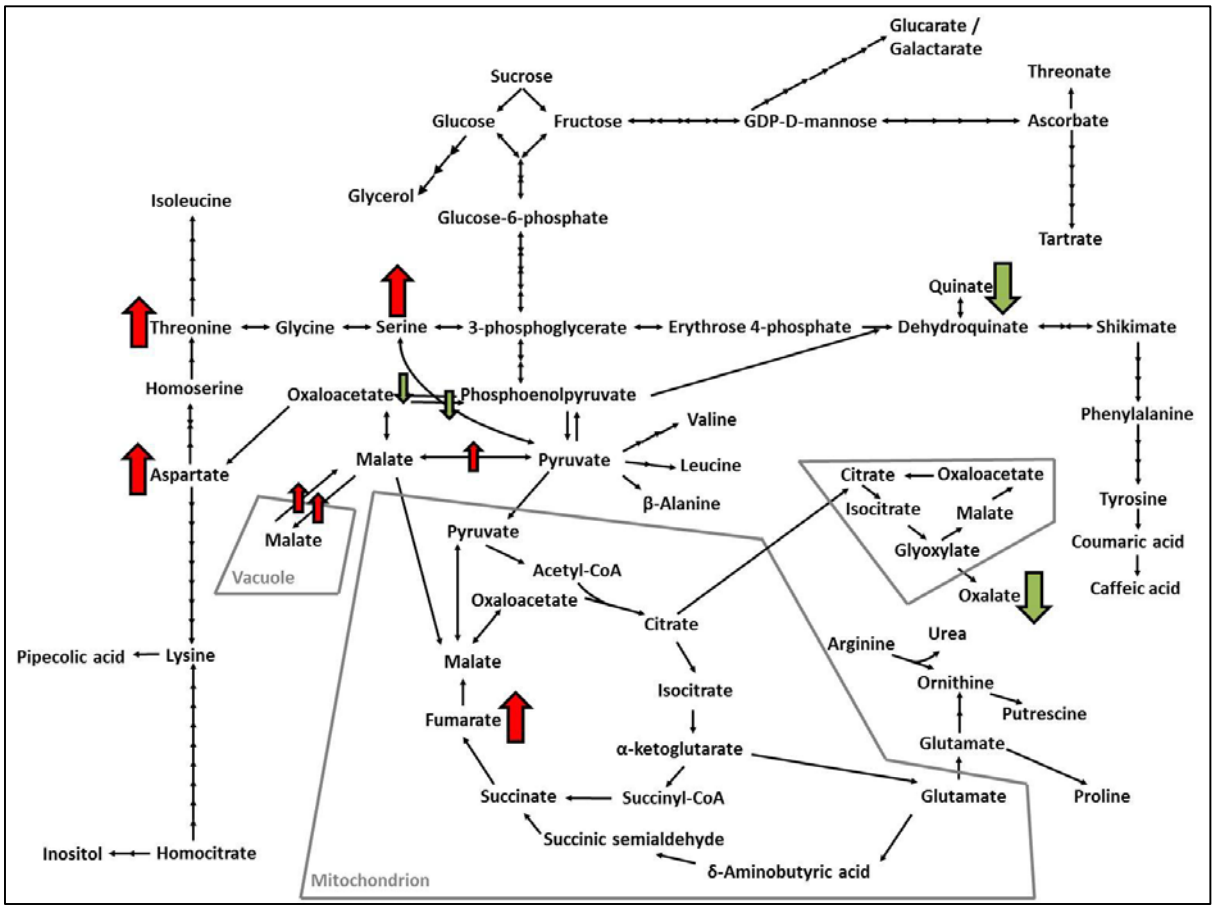


Figure 29: Chenin Blanc ripening transcript and metabolite model. Large arrows represent increases (up-facing, red arrow) or decreases (down-facing, green arrow) in metabolite levels relative to other cultivars. Smaller arrows represent increases or decreases in transcript levels. (n = 4 ± S.E.M.).

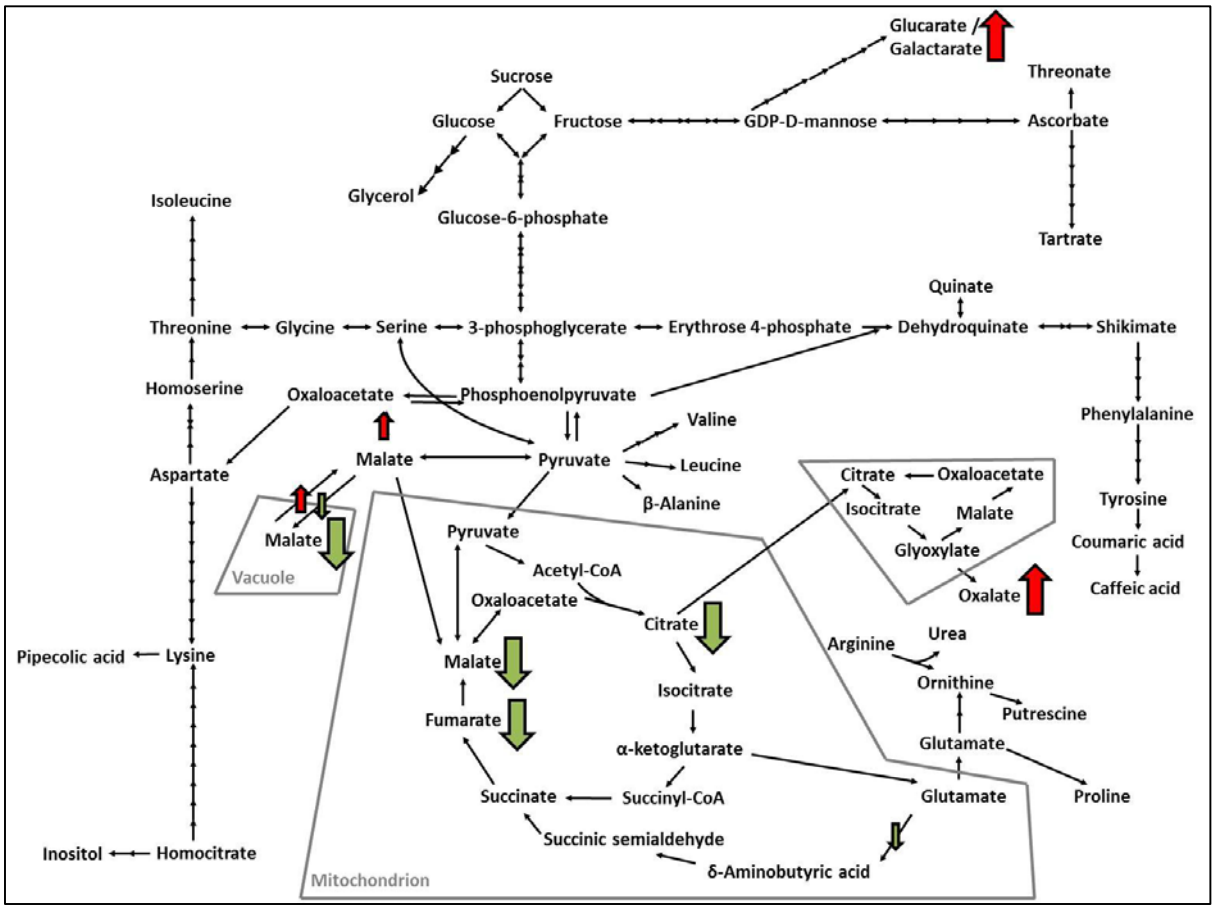


Figure 30: Palomino ripening transcript and metabolite model: Large arrows represent increases (up-facing, red arrow) or decreases (down-facing, green arrow) in metabolite levels relative to other cultivars. Smaller arrows represent increases or decreases in transcript levels. (n = 4 ± S.E.M.).

6.4. Output 4

Effect of temperature on cytosolic pH and malic acid concentration in various red fruit cultivars.

Excised canes from field-grown grapevines were used to compare a variety of *V. vinifera* cultivars for heat response with respect to malic acid concentration in the berry, as well as an estimate of cytosolic pH within berry slices. During the fruit ripening stage, full-length canes containing single bunches were excised from field-grown vines and the excision sites were submerged in a quarter- strength MS media containing 3 % (w/v) sucrose, 0.1 % (w/v) casein hydrolysate and 40 ppm sodium hypochlorite. The canes were fastened into an upright position within Conviron cabinets at The Plant Accelerator, The University of Adelaide, Urrbrae, South Australia, with daily media changes. Canes were kept for two days at control temperature conditions (ramped cycles: 25°C maximum, 15°C minimum) before dividing into two groups, subjecting one group to elevated temperature conditions (ramped cycles: 35°C maximum, 20°C minimum) and the other maintained at control temperature conditions.

Subsets of berries were harvested and snap-frozen for fresh weight and HPLC analysis, or used immediately for Brix measurements. Additional berries were also used for immediate *in vivo* cytosolic pH measurements with fresh tissue (Figure 31).

The five-day elevated temperature treatment did not significantly affect berry size, although a slight trend toward smaller berries was seen in most cultivars except Barbera, which showed slightly larger berries (Figure 31a). Total soluble solids were generally slightly, but not significantly higher in the heat-treated fruit, indicating either an advancement of ripening or a concentrating effect due to smaller berry size (Figure 31b). Malic acid concentrations were generally lower in the heat-treated fruit, although not significantly except in the case of Sangiovese and Shiraz cultivars (Figure 31c). Cytosolic pH did not show significant differences between control- and heat-treated fruit. However, there were some differences between cultivars. In particular, cultivars that had lower malic acid levels seemed to have lower cytosolic pH, by as much as pH 0.5 (Figure 31d).

Tinta Cao berries at two different stages (véraison and ripening) were analysed separately in this experiment, and it was observed that the véraison fruit had a lower cytosolic pH (Figure 32). The difference in pH was approximately 0.4 units. To look more closely at this, berries from Shiraz vines, as well as the four cultivars described in Output 3 (Palomino, Chenin Blanc, Cabernet Sauvignon and Tinta Cao) were collected at various stages throughout development from field-grown vines and used for cytosolic pH estimations (Figure 33). All cultivars showed a slight decrease in cytosolic pH at the colour change, relative to earlier stages; statistically significant for Tinta Cao (P=0.05), Chenin Blanc (0.002) and Palomino (0.02). Chenin Blanc demonstrated the largest change, with a decrease from pH 7.06 at softening, to pH 6.65 at the colour change. This difference may indicate a temporary

acidification of the cytosol at véraison, and may be related to the release of organic acids from the vacuole at this time. A more thorough exploration of cytosolic pH changes during berry development is warranted, with further optimisation of new technique to enable the analysis of mature berries.

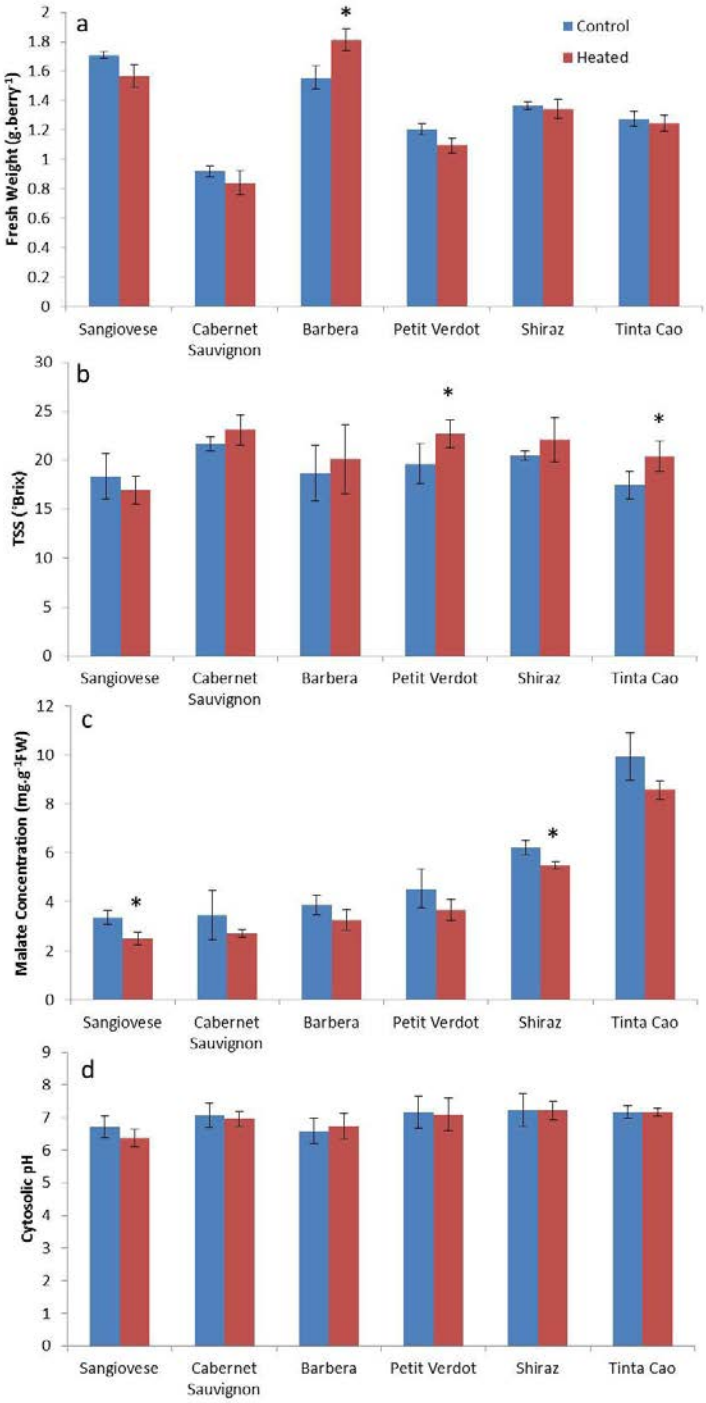


Figure 31: Excised canes subjected to 5 days of elevated temperature conditions within growth cabinets. (a) Berry fresh weight (n = 4), (b) Total Soluble Solids (n = 8), (c) malic acid concentration (n = 4) and (d) estimation of cytosolic pH. * P<0.05 based on two-tailed t-tests.

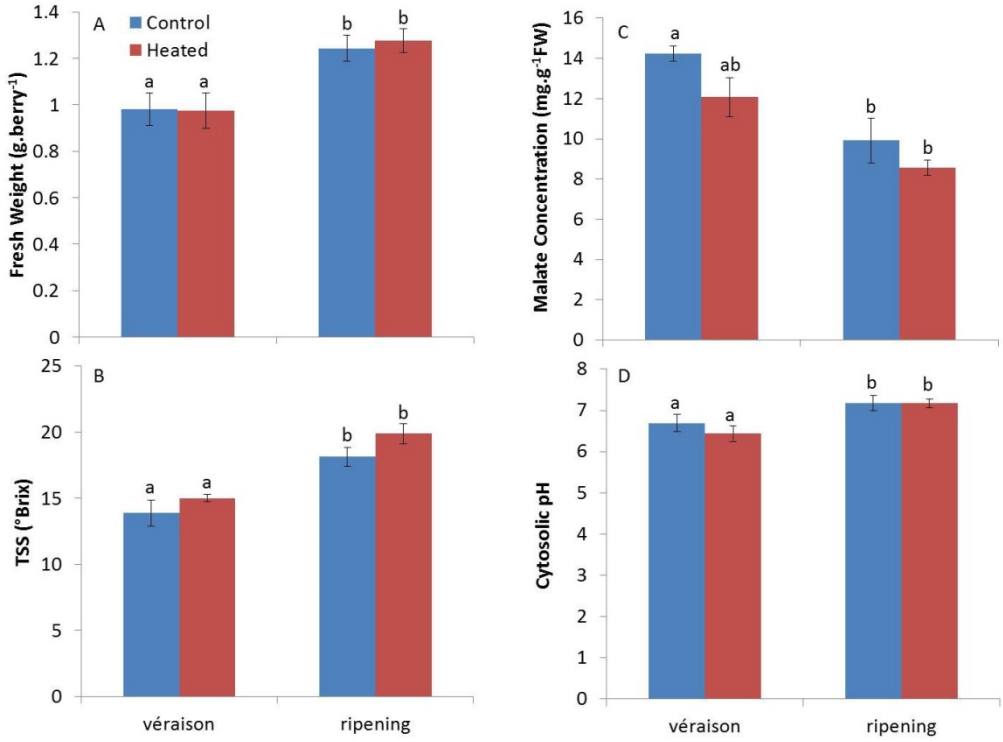


Figure 32: Véraison and Ripening berries of Tinta Cao from growth cabinet heat treatment study. a) berry fresh weight (n = 4 ± S.E.M), b) total soluble solids (n = 4 ± S.E.M), c) malate concentration (n = 4 ± S.E.M) and d) cytosolic pH (n = 7 ± S.E.M.). Lower-case letters indicate significant differences based on ANOVA with multiple comparisons t-tests.

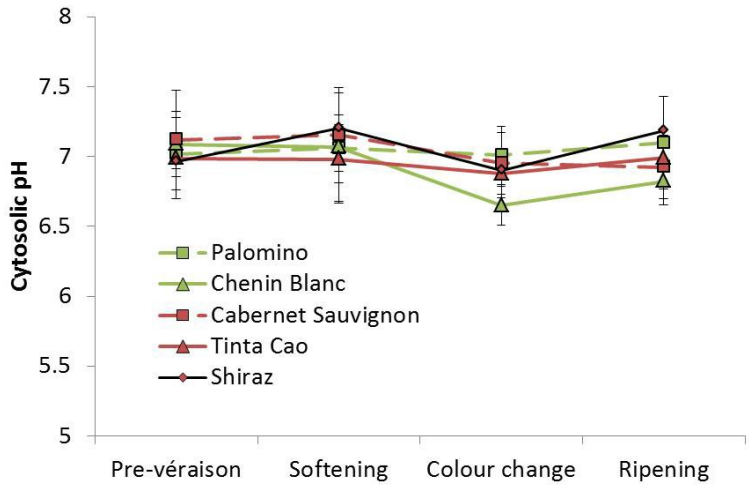


Figure 33: Cytosolic pH of four cultivars throughout development and ripening. "Pre-véraison" berries were those that were still hard, but came from bunches where other berries were beginning to soften. "Softening" berries were those that had begun to soften but were still green. "Colour change" berries were those where approximately 50% of the skin contained anthocyanins, or for non-pigmented berries the colour change was determined as skins that demonstrated some transparency. "Ripening" berries were selected from bunches where average TSS were approximately 13°Brix. Mature berries were more difficult to analyse due to the fragility of the berry slices. (n = 7 ± S.E.M.).

6.5. Output 5

Characterisation of tartaric acid biosynthesis pathway candidate enzymes

Enzymes of the putative pathway of tartaric acid have been outlined elsewhere (DeBolt *et al.*, 2006; Burbidge, 2011) and are summarised in Figure 34.

In previous work, an L-idonate dehydrogenase (Step 3 in Figure 34) was recombinantly expressed and shown to catalyse the conversion of L-idonate to 5-keto-D-gluconic acid (DeBolt *et al.*, 2006). Since then we have identified two more putative L-idonate dehydrogenase genes, as well as potential candidate genes for the 2-keto-L-gulonate reductase (step 2a in Figure 34), the transketolase (step 4 in Figure 34) and the succinic semialdehyde dehydrogenase (step 5 in Figure 4). We attempted to confirm the activities of these gene products by recombinant expression and enzyme assay kinetics, as well as activity assays from crude grape berry extracts.

6.5.1. 2-Keto L-gulonic acid reductase (2-KGR)

A gene putatively encoding this enzyme (Accession: XM_003632812) was previously isolated from grape, expressed in bacteria and shown to utilise the predicted substrate, 2-keto-L-gulonic acid (Burbidge, CA, PhD Thesis). To further this work, the resultant product was analysed using GC/MS to determine whether it was the next expected intermediate of the tartaric acid biosynthesis pathway, L-idonate. Authentic standards of 2-keto-L-gulonate and L-idonate were resolved using this method. The standard solution of 2-keto-L-gulonic acid gave multiple peaks on the chromatogram at 5.73, 5.95, 6.08 and 6.18 minutes (Figure 35a). The standard solution of L-idonate gave a major peak at 6.43 minutes, and also a very small peak at 6.04 minutes (Figure 35b). Upon addition of 2-KGA to an assay mix containing the 2-KGR enzyme and NADH in Tris buffer, pH 7.5, the resultant product demonstrated the major and minor peaks characteristic of L-idonate at 6.43 and 6.04 minutes, as well as some residual peaks characteristic of 2-KGA (Figure 35c). There was an additional large peak at 2.50 minutes that was not present in either of the standard solutions, and which also appeared in the boiled enzyme control (Figure 35e), therefore this likely represents a non-enzymatic breakdown product of 2-KGA. When the same assay was run in the presence of NADPH instead of NADH, a similar chromatogram was observed (Figure 35d). The mass:charge ratios for the peaks observed at 6.43 and 6.04 minutes for the enzyme assay products were the same as those observed for the authentic L-idonate standard (Figures 36 and 37). Therefore this enzyme can convert 2-keto-L- gulonate to L-idonate, and thus could serve as the 2-KGR step of the tartaric acid biosynthesis pathway in *V. vinifera*.

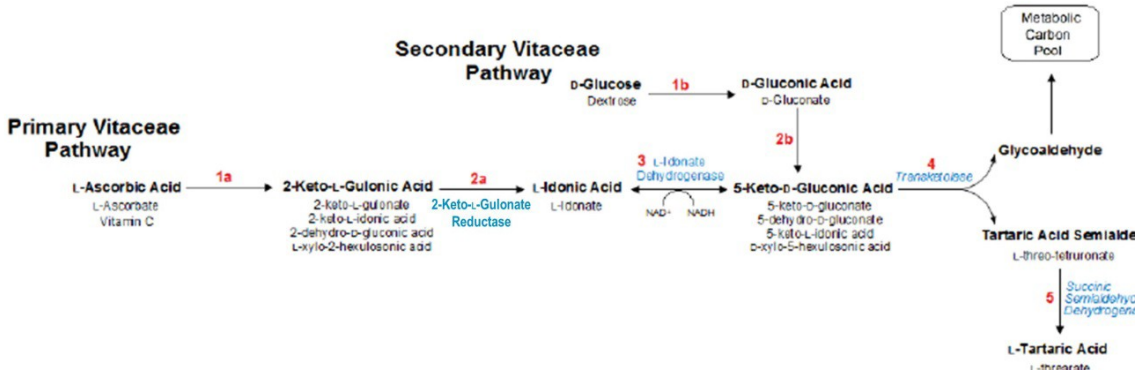


Figure 34: Potential pathways of tartaric acid biosynthesis in Vitis vinifera. Demonstrates the metabolic intermediates involved in the conversion of ascorbate or glucose to tartrate (black), and putative enzymes with the potential to catalyse the various steps (blue). Image derived from Burbidge (2011).

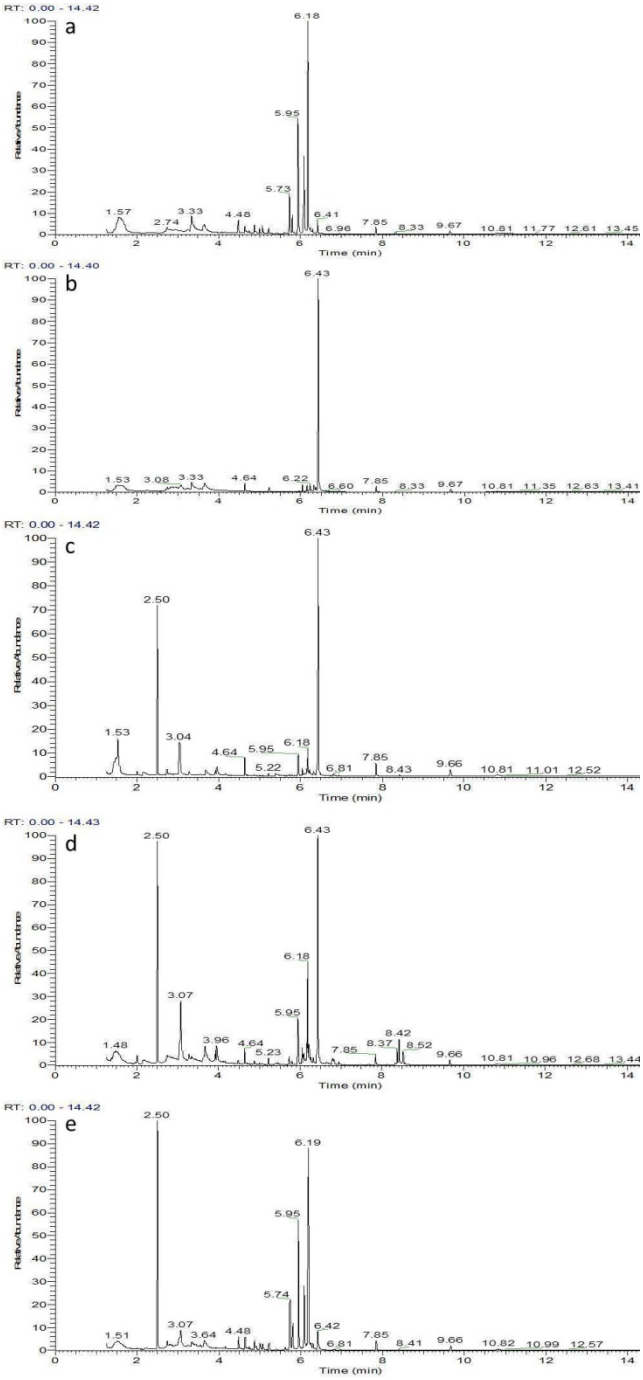


Figure 35: Chromatograms from 2-KGR enzyme assay products and authentic standards. a) a standard solution of the substrate 2-keto-L-gulonate, b) a standard solution of the expected product L-idonate, c) product of the 2-KGR activity assay in the presence of NADH, d) product of the 2-KGR activity assay in the presence of NADPH, e) product of the 2-KGR assay in the presence of NADH and boiled enzyme. NADH (or NADPH) consumption was visualised using a spectrophotometer, to monitor the progress of the reaction before freeze-drying the product and preparing for GC-MS analysis. The typical peak for L-idonate at 6.43 minutes was observed in the two assays containing purified enzyme in the presence of NADH or NADPH (c, d). An additional product was observed at 2.5 minutes in the presence of purified enzyme and boiled enzyme, suggesting some non-enzymatic breakdown products of 2-KGA under these assay conditions.

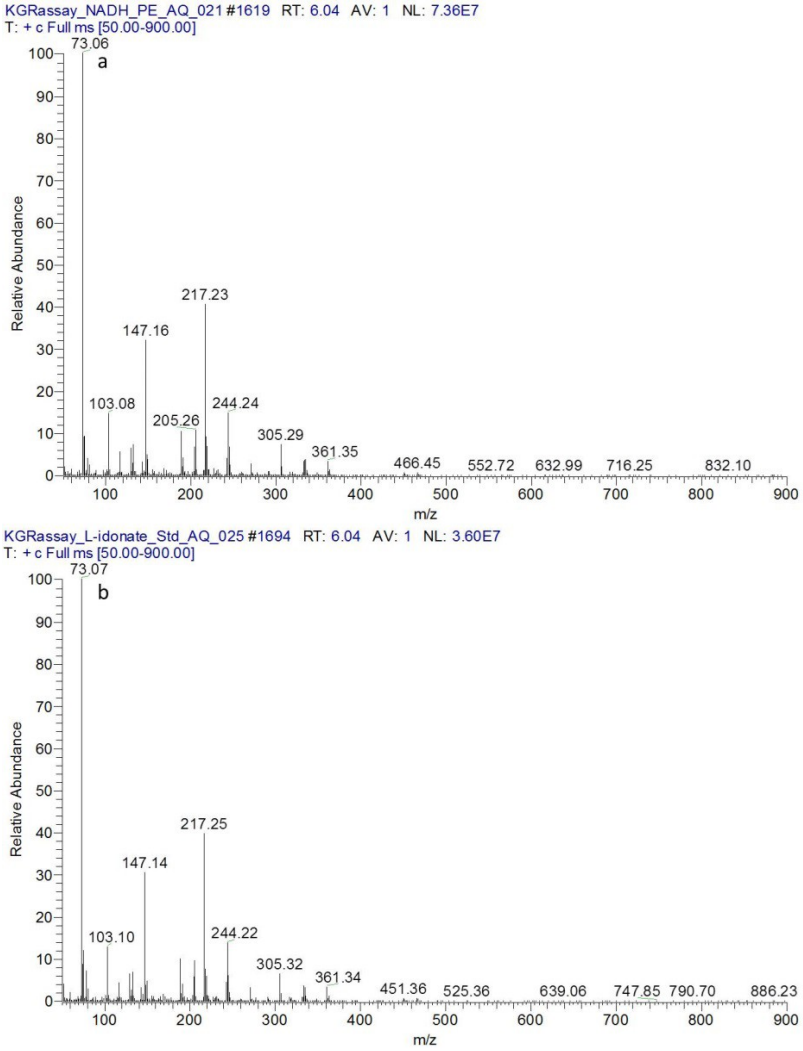


Figure 36: Mass/charge profiles of minor L-idonate peak (6.04 mins). (a) assay product and (b) authentic standard.

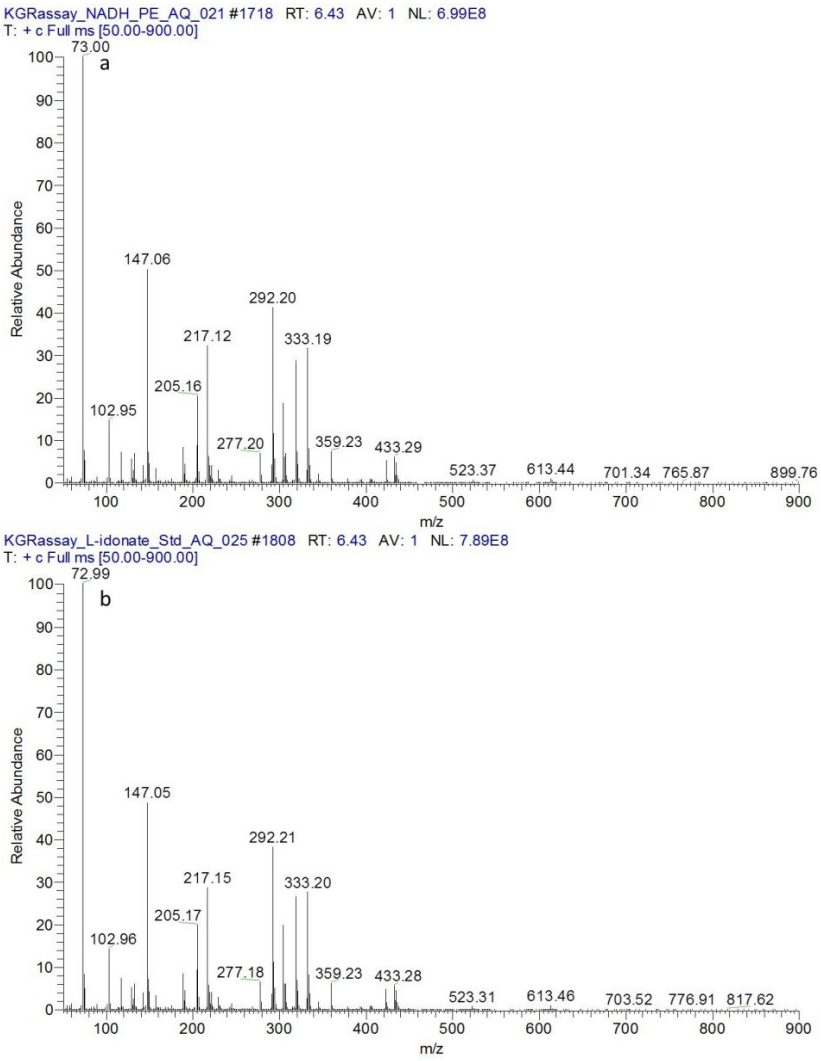


Figure 37: Mass/charge profiles of major L-idonate peak (6.43 mins). (a) assay product and (b) authentic standard.

Although the enzyme was shown to successfully convert 2-keto-L-gulonate to L-idonate, there remained some uncertainty about the substrate specificity of the expressed enzyme, since the sequence showed similarity to other dehydrogenases that use several other substrates. Therefore, additional substrates were tested.

In an initial screen, approximately 4-5 fold greater activities were observed with 1mM glyoxylate when compared to 10 mM 2-KGA. With 2-KGA, assays also showed some non-linearity, suggesting an inhibitory component, or substrate inhibition. With a range of substrate concentrations, as a preliminary to kinetics experiments, the Km for 2-KGA was approximately 5mM, whereas that for glyoxylate was less than 0.5mM. With NADPH (2mM), activities with both substrates were higher, by approximately 3-fold with 2-KGA. Subsequent freezing and thawing of the enzyme preparation led to large loss of activity, therefore freshly purified enzyme was subsequently stored in single-use aliquots for more thorough kinetic analysis.

Results of the kinetic analysis are demonstrated in Table 2. In the presence of 2-KGA and either NADPH or NADH, the enzyme demonstrated reasonable Vmax's of 7 $U.mg^{-1}$ and 17 $U.mg^{-1}$ and low K_ms of 0.7 mM and 1.56 mM, respectively. However, activity with glyoxylate in the presence of NADPH and NADH showed high Vmax' of 69 and 14 $U.mg^{-1}$ and very low K_ms of 0.04 mM and 0.25 mM, respectively. The enzyme also showed a high affinity for hydroxypyruvate in the presence of NADPH (K_m 0.1 mM), but with a lower Vmax, and no activity was observed when NADPH was replaced by NADH. 2-KGR also demonstrated low rates of activity and high K_ms with pyruvate. Based on the Kcat and relative Vmax/Km values, the preferred substrate for the enzyme is glyoxylate. Hydroxypyruvate was also a good substrate, with 18% relative Vmax/Km when compared to that of Glyoxylate+NADPH. 2-KGA also showed a decent rate of activity, but with lower affinity than glyoxylate and hydroxypyruvate. Therefore, the enzyme is likely a glyoxylate reductase that may act as a hydroxypyruvate reductase or 2-keto-L-gulonate reductase in the presence of sufficient substrate.

Table 2: Kinetic properties of recombinantly-expressed *V. vinifera* 2-Keto-L-gulonate reductase.

Substrate	K_m (mM)		V_{max} (U.mg^{-1})		K_{cat} (.sec^{-1})		Rel. V_{max}/Km (%)	
	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH
2-keto-L-gulonate	1.56	0.70	17.19	7.54	10.01	4.31	3.25	3.12
Glyoxylate	0.25	0.04	69.38	13.82	39.67	7.90	80.32	100
Hydroxypyruvate	-	0.10	-	6.30	-	3.60	-	18.23
Pyruvate	3.45	1.37	0.44	0.55	0.25	0.31	0.04	0.12

6.5.2. Transketolase

A transketolase-type enzyme activity has been postulated to convert 5-keto D-gluconic acid (5-KG to tartaric acid semialdehyde in the suggested pathway of TA biosynthesis. To test this, two grape transketolase genes were isolated and expressed in a bacterial heterologous expression system. Transketolase genes I and II were cloned into the expression vector pET14b and expressed in *E. coli*. Extracts were prepared on several occasions and the expressed protein purified using Ni-His affinity chromatography columns.

Several different spectrophotometric assays were attempted to demonstrate activities with 5-keto-D-gluconate, as an intermediate of the tartaric acid biosynthesis pathway, as well as alternative substrates. These assays were generally made difficult by the poor availability of substrates (erythrose-4-phosphate and xylulose-5-phosphate), and a source of transketolase to use as a positive control. The assays rely on coupling systems to measure the products; either glyceraldehyde-3-phosphate (GAP), or fructose 6-phosphate (F6P). While the assay coupling systems could be demonstrated to be effective, various attempts to demonstrate control transketolase activity from bacterial or plant sources were unsuccessful. Eventually, a control activity of transketolase could be best shown using fresh spinach extracts in an indirect assay, by coupling with ribose-5-phosphate isomerase and ribulose-5-phosphate epimerase (from the extract) to produce xylulose-5-phosphate. This was followed by the addition of ribose-5-phosphate, with the products GAP and F6P measured using glycerol phosphate dehydrogenase/triose phosphate isomerase, or phosphoglucose isomerase/glucose- 6-phosphate dehydrogenase, respectively. Some details and results using this assay are summarised below.

Fresh baby spinach leaves were obtained from a local green grocer shop and kept at room temperature in the dark overnight. Leaf tissue (2g, with main vein taken out) was homogenised in an ice-cold mortar and pestle with 4 mL of 50 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM TPP (Buffer A). Approximately 3 mL of the slurry was centrifuged at full speed (16,000 g) for 6 min. the supernatant was directly applied to a PD10 column (G-25) previously equilibrated with buffer A, and approximately 1.5 mL of the protein band collected.

Assays were carried out in 1 mL cuvettes at 25°C in a Dynamica DB20 spectrophotometer at 340 nm, by following the decline in NADH absorption. The reaction mix contained 40 mM Tris-Cl buffer, 4 mM $MgCl_2$, 0.08 mM TPP, 0.2 mM NADH, 2.0 mM ribose-5-phosphate (R-5-P), 1.0 mM xylulose-5-phosphate (Xu-5-P), as well as triose phosphate isomerase, glycerolphosphate dehydrogenase and spinach extract (25-50 µL).

A rate of 0.556 OD/min/50 ul was obtained in the presence of both substrates. With only R-5P, or with only Xu-5-P, rates of 0.409 or 0.159 OD/min/50ul were obtained, respectively. In each case there was a lag before achieving the maximum rate, suggesting that there was partial inter- conversion of each of the substrates to the other via ribose-5-phosphate isomerase and ribulose-5-phosphate epimerase. Background activity in the absence of both substrates was 0.006 OD/min/50ul. These results confirmed the presence of transketolase activity in the spinach extract.

Using this assay and samples of expressed transketolase protein, there was negligible activity for transketolase or 5-KG conversion. The coupling system was checked and shown to be very active by addition of glyceraldehyde-3-phosphate to the mix.

In addition, there was also no activity using an alternative assay with L-erythrulose, ribose-5- phosphate, and alcohol dehydrogenase coupling (based on the method of (Hecquet *et al.*, 1993), using the expressed protein. In this case the coupling system was shown to be active by adding acetaldehyde to the mix. In the case of plant extracts, although low activities were observed on some occasions, activity with this L-erythrulose assay is very low, presumably due to competition of the weakly binding L-erythrulose by the more strongly binding Xu-5-P formed in the reaction mix.

The results overall show that the grape enzyme as expressed in pET 14 was not active as a transketolase. Subsequent sequencing showed the expression constructs to have the correct sequence and start sites. To prove that the grape transketolase could have the capacity for 5-keto- glutarate conversion, active protein may need to be derived from alternative expression constructs or expression systems.

6.6. Output 6

Grapevines with altered expression of genes involved in malic acid and tartaric acid biosynthesis and catabolism.

To test the effects on tartaric acid biosynthesis and malic acid metabolism of modifying some of the enzymes mentioned in previous outputs, a genetic approach was taken. Due to its short reproductive cycle and therefore reduced timing required for development of testable transgenic grapevine material, the microvine was selected as a model system for transformation experiments. Despite their unusual physiology (whereby multiple bunches develop at different times along a single cane), microvines produce berries that demonstrate typical patterns of malic and tartaric acid accumulation during development (Figure 38).

Microvine embryos were transformed using Agrobacterium cultures harbouring the vectors of interest. Plants were regenerated on selection media, transferred to soil, and grown in The Plant Accelerator, The University of Adelaide, Urrbrae, South Australia. In total, 36 plants were regenerated from the *VvLidh1* RNAi transformation (two rounds of transformations were conducted for this construct), four plants for *VvLidh1* overexpression, eight for *VvLidh2* and eight for *VvLidh3* overexpression. In addition, seven plants were obtained from the *Vv2Kgr* RNAi transformation, eight from the *VvStop1* overexpression transformation and twelve from the *VvGalur* overexpression transformation. Unfortunately, while some plants were cultured following the *VvNadme1* and *VvPepc2* transformation, these plants did not survive the transfer to soil. All surviving plants are summarised in Table 3.

Leaf tissue was collected from each plant (T1 generation) and genomic DNA screened for the presence of the appropriate cassettes in each line. Genomic DNA was extracted from 83 microvine plants in total, containing the various constructs. DNA samples were checked for quality using PCR amplification of the actin gene. All successful transgenic lines should contain a cassette that includes the CaMV35S promoter. Therefore, initially each sample was screened using a forward and reverse primer combination that targeted the CaMV35S promoter. Many of the DNA samples demonstrated products of the expected size, including six of the L-IDH RNAi lines, six of the STOP1 overexpressor lines, two of the GalUR overexpressor lines and three of the 2-KGR RNAi lines (Table 3). Subsequently, PCR assays specific to each construct were designed for further screens.

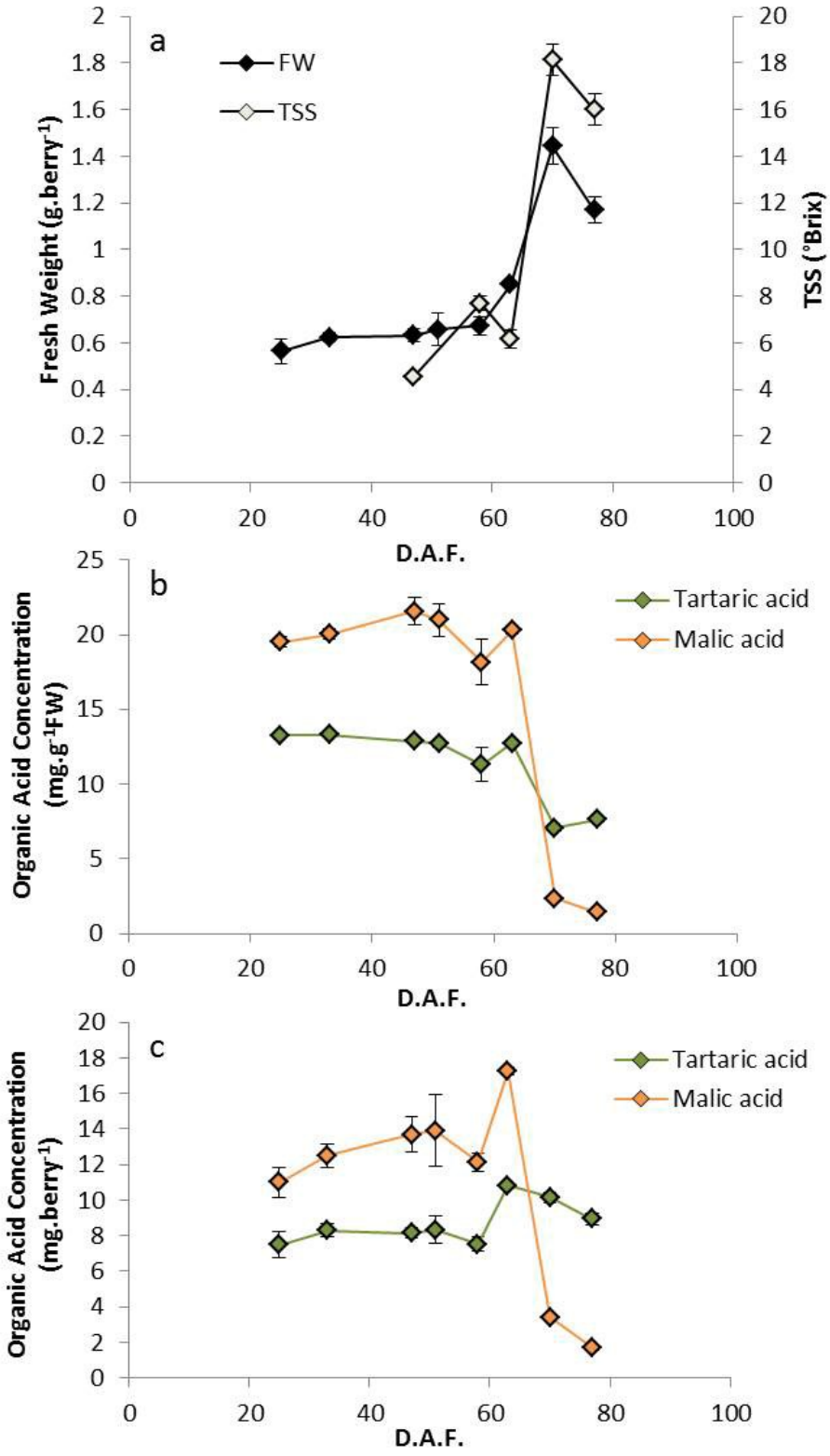


Figure 38: Normal developmental properties of microvine berries (a) fresh weight and Total Soluble Solids (TSS), (b) malic and tartaric acids presented as mg.gFW^{-1} and (c) malic and tartaric acid concentrations presented as mg.berry^{-1}. Véraison occurred at approximately 65 days after flowering (n = 3 ± SEM).

Table 3: Summary of transgenic microvines available for the exploration of altered genes of TA and MA metabolism. The numbers of individual T1 plant lines obtained by genotyping analyses, using Hyg^R: selection based on Hygromycin resistance, CaMV35S: successful PCR amplification of the Cauliflower Mosaic Virus promoter region from the expression cassette, and GSP: successful PCR amplification using Gene-Specific primers in combination with Promoter-Specific or Recombination-site-Specific primers.

Target	Accession	Construct	Hyg^R	CaMV35S	GSP
L-idonate dehydrogenase 1 RNAi knockdown	XM_002269900	pHellsgate-IDH1	24	18	0
L-idonate dehydrogenase 1&2 RNAi knockdown	XM_002269900 XM_002269859	pHellsgate-IDH1&2	12	6	0
L-idonate dehydrogenase 1 overexpression	XM_002269900	pBMTh2-IDH1	4	4	2
L-idonate dehydrogenase 2 overexpression	XM_002269859	pBMTh2-IDH2	8	7	5
L-idonate dehydrogenase 3 overexpression	XM_002267626	pBMTh2-IDH3	8	8	7
2-ketogluconate reductase RNAi knockdown	XM_003632812	pHellsgate-2KGR	7	7	6
galacturonate reductase overexpression	NM_001281196	pCLB1301-GalUR	12	10	8
Stop1 overexpression	XM_002270160	pCLB1301-STOP1	8	7	4

For the 2-KGR RNAi lines, a forward primer targeting the CaMV35S promoter in the pHellsgate12NH vector and a reverse primer designed to the 2-KGR gene RNAi target sequence were used. These primers had been previously shown to amplify a product of approximately 920 bp from the plasmid DNA used to generate the transgenic microvine lines. Using these primers, 6 out of the 7 potential 2-KGR RNAi lines contained the construct.

For the L-IDH RNAi lines, the same strategy was used to screen for an expected product of 555 bp. While many of these samples showed amplification of the CaMV35S promoter, none showed amplification products for the L-IDH RNAi cassette. The positive control using vector DNA showed a strong product. Therefore, either these lines did not successfully integrate the whole L-IDH RNAi cassette into the genome as part of the RNAi cassette, or amplification of the RNAi cassette from genomic DNA is inhibited (e.g. secondary structures that may interfere with primer binding).

For the L-IDH overexpressor lines, the same strategy was used, except the F primer was designed to target the Gateway recombination site (att site) in the cassette and the reverse primers were each designed to specifically target one of the three *VvLidh* genes. Each cassette was screened using two or three different primer sets, and checked against a wildtype microvine gDNA sample to ensure that there was no non-specific binding within the microvine genome. Two plants contained the overexpression cassette for *VvLidh1*, five plants for *VvLidh2* and seven plants for *VvLidh3*.

For the STOP1 overexpressor lines, a forward primer targeting the CaMV35S promoter in the pCLB1301 vector and reverse primers targeting the STOP1 gene were used. Three primer sets were used, with expected product sizes of 770, 856 and 962. Four plants potentially contain this overexpression cassette.

For the GalUR overexpressor lines, the same strategy was used, with expected product sizes of 864, 916 and 1005 bp. Eight plants potentially contain this overexpression cassette.

Figure 39 illustrates examples of plants in tissue culture, after transplanting into soil and once they began producing berries. Due to the use of a constitutive promoter in the constructs, effects may be expected within all plant tissues. Leaf and berry tissues from these plants are currently being collected and tested for organic acid levels at various stages of development.



Figure 39: Images of various transgenic microvines housed at The Plant Accelerator.

6.7. Output 7

Metabolic analysis of berries naturally lacking tartaric acid.

The wild vine *Ampelopsis aconitifolia* naturally lacks tartaric acid and the L-idonate dehydrogenase gene transcript. In previous work, the berries demonstrated extremely high levels of ascorbate (DeBolt et al. 2006), a precursor of tartaric acid biosynthesis. In the current work, metabolic profiling of ripening berries of *A. aconitifolia* demonstrated extremely high levels of quinate; 70-fold higher than berries of *V. vinifera* (Figure 40). Quinate is formed from dehydroquinate, which can arise from erythrose-4-phosphate and PEP from the pentose phosphate pathway and glycolysis respectively, or from the shikimate pathway. Erythrose-4-phosphate, as mentioned previously, is a substrate or product of transketolase in the pentose phosphate pathway or Calvin cycle, respectively. In the absence of L-idonate dehydrogenase activity, a transketolase that could otherwise be employed to convert 5-keto-L-gulonate to tartaric semialdehyde in *V. vinifera* may instead over-produce erythrose 4-phosphate, with an end-point accumulation of the astringent, quinate, which can be a storage pool for carbon in some plants (Grace and Logan, 2000).

Considering that the precursor for tartaric acid biosynthesis in grapes is ascorbate, and that when tartaric acid cannot accumulate, the fruit accumulate quinate instead, tartaric acid in leaves and berries may act as an overflow storage pool for oxidised antioxidant metabolites that, in berries at least, may occur during early development when cell growth and division generates high levels of reactive oxygen species (ROS). High levels of ROS can induce glutamate dehydrogenase expression within grapevine cells (Skopelitis *et al.*, 2006), which may explain the high level of glutamate in *A. aconitifolia* berries that lacked tartaric acid. As levels of proline and putrescine were very low and GABA was virtually unchanged in comparison to *V. vinifera* berries, the extra glutamate was not used to enhance these stress-inducible metabolites.

The berries of *A. aconitifolia* were physiologically and metabolically dissimilar to those of *V. vinifera*, based on visible differences in the size, shape and colour, as well as bland taste and milky, astringent texture. It is unlikely that the lack of tartaric acid biosynthesis is the sole cause of these differences. For example, this species also demonstrated an almost complete lack of malic acid, as well as increased levels of citrate, succinate and glutamate, suggesting that the TCA cycle was interrupted, potentially leading to the low observed levels of amino acids (valine, leucine, isoleucine, serine, threonine). Nevertheless, tissues or cell cultures from this species could provide a useful model for confirming the role of various candidates of tartaric acid biosynthetic enzymes through complementation studies.

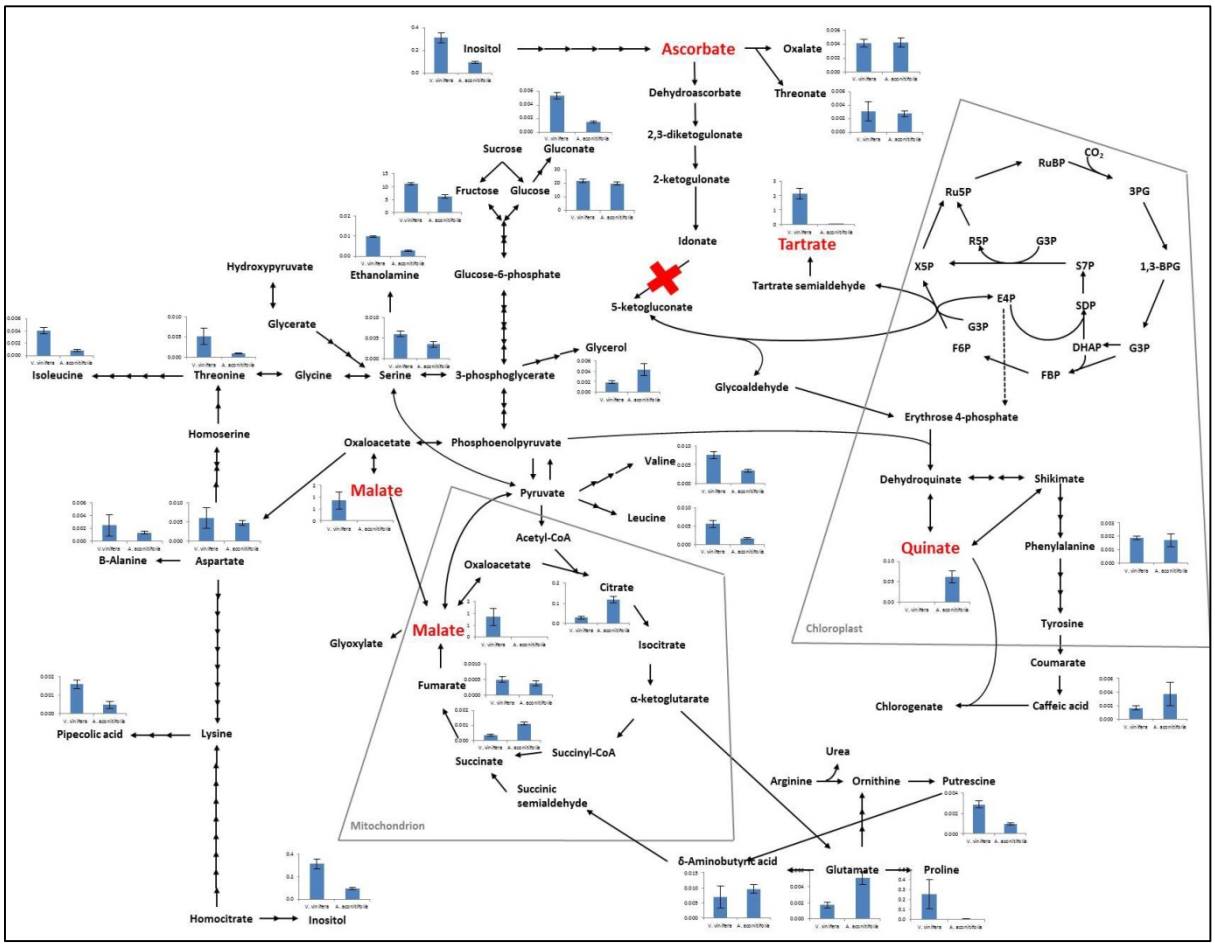


Figure 40: Metabolic map of *A. aconitifolia* berries and *V. vinifera* berries. Graphs indicate levels of the indicated metabolite (relative to ribitol internal standard) in *V. vinifera* (left) and *A. aconitifolia* (right). Metabolites of the Calvin cycle are abbreviated as RuBP: Ribulose-1,5-bisphosphate, 3PG: 3-phosphoglycerate, 1,3-BPG: 1,3-bisphosphoglycerate, G3P: glyceraldehyde-3-phosphate, FBP: fructose bisphosphate, F6P: fructose-6-phosphate, X5P: xylose-5-phosphate, Ru5P: Ribulose-5- phosphate, DHAP: dihydroxy-acetone-phosphate, SDP: sedoheptulose-1,7-bisphosphate, S7P: sedoheptulose-7-phosphate, E4P: erythrose-4-phosphate, R5P: ribose-5-phosphate. Compartmentalisation is shown where possible, but may not accurately depict localisation of all metabolites. (n = 4 ± SEM).

6.8. Output 8

Leaf-based systems to identify and characterise genes and enzymes involved in TA and MA metabolism

Leaves were sampled from the Coombe vineyard, University of Adelaide Waite Campus, South Australia (35˚S, 139°E) during the 2010-11 and 2012-13 seasons, for investigation of organic acid levels.

During the 2010-11 season, leaf samples were collected alongside berry samples, therefore organic acid accumulation was measured in the leaves throughout the berry development and ripening stages. Malate and tartrate showed typical accumulation patterns in Semillon fruit, with both acids accumulating during development and malate decreasing during ripening. In the leaves, malate levels changed little, but were of similar levels to those seen in young (and ripening) berries, while tartrate levels of the leaves were over three times as high as levels seen in the berry (Figure 41).

During the 2012-13 season a different approach was taken in order to analyse the organic acids of grapevine leaves at different leaf ages, rather than at different stages of berry development. That is, leaves from a cane were harvested at different nodes of the cane, based on the date on which the leaves opened. Again, the levels of tartaric acid were higher than malic acid in the leaves of Semillon grapevines, which reached 15 and 4 mg.g-1FW, respectively, following steady increases throughout leaf ageing (Figure 42). However, the acids of leaves at Node 11 were lower than they were at any stage during the 2010-11 season. Therefore there may be seasonal differences in tartaric acid accumulation in leaves of grapevine.

There was no degradation phase for malic acid during leaf development and therefore the leaf was considered impractical as a system for studying berry malic acid metabolic pathways. However, leaves may prove useful for further study of berry tartaric acid metabolism, as high levels of tartaric acid were consistently accumulated throughout development.

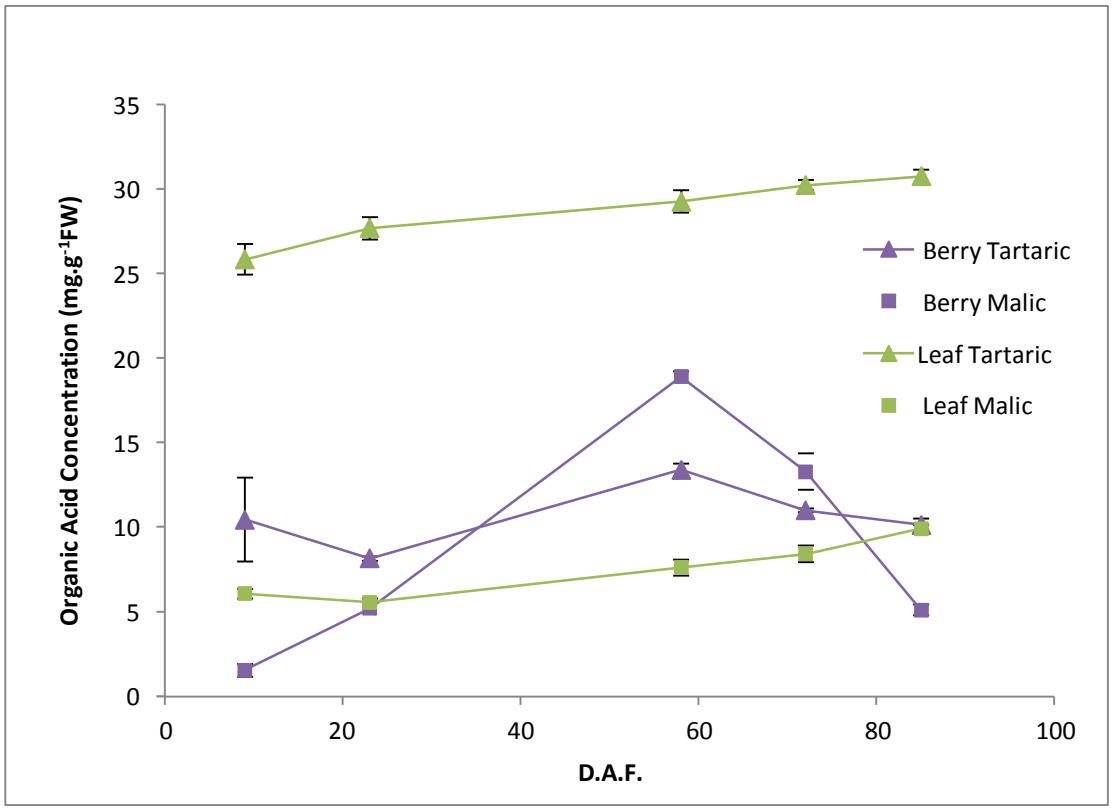


Figure 41: Semillon leaf and berry organic acids during berry development and ripening. Samples were collected during the 2010-11 season. Bunches of Semillon fruit were tagged at anthesis and used for fruit collection at regular intervals (2-3 times per week). Leaves were also collected at particular stages of berry development, on 23 November 2010 (young berries) and 7 December 2010 (pre-véraison), 11 January 2011 (véraison), 25 January 2011 (ripening) and 7 February 2011 (ripe berries). Leaves were taken from canes that did not contain tagged bunches, and were selected based on similar size and similar age (always from node 11). (n = 4 ± S.E.M.).

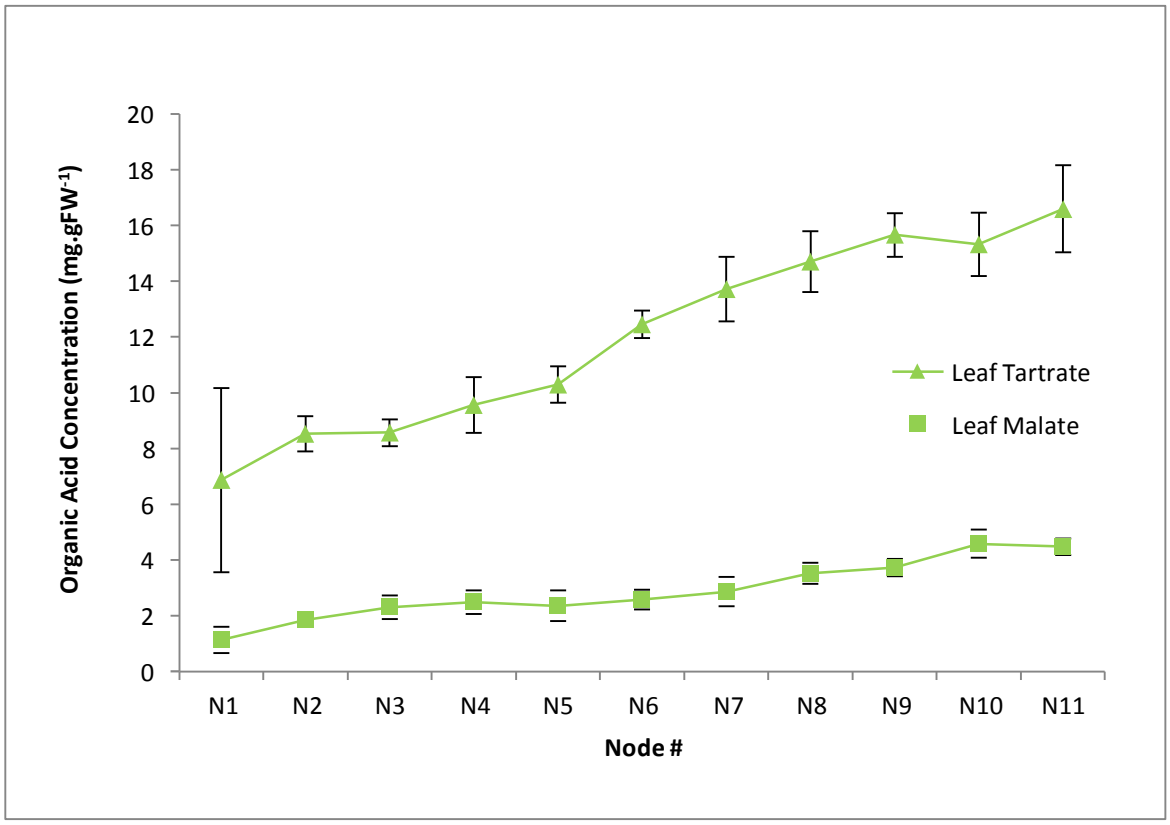


Figure 42: Semillon leaf organic acids based on leaf age. Samples were collected during the 2011-12 season. After budburst, sixteen canes were selected across four Semillon vines (i.e. four replicate canes per vine), and the leaves of those canes were tagged as they unfolded (approximately two leaves per week). Once the cane reached least 11 nodes, each tagged leaf was collected. The collection date occurred when the berries of the vine were at the post-véraison stage on 24 January 2013 (which most closely aligns with the 72 D.A.F. timepoint from the 2010-11 experiment). (n = 4 ± S.E.M.)

7. Outcome/Conclusion

The two main practical aims as outlined in Section 2: Executive Summary, were to:

1) Identify important regulatory junctions of malic acid metabolism that may be targeted to reduce acid losses during hot periods of the season.
2) Discover new genes involved in the largely uncharacterised tartaric acid biosynthesis pathway, such that tartaric acid production may be manipulated to control acidity in the berry regardless of seasonal temperature.

To address these aims, a number of smaller aims and outputs were outlined in Section 4: Project Aims and Performance Targets. These are dealt with individually, below.

Aim 1: To show how grapevines respond to growth under warming temperatures and to extreme temperature events with respect to organic acid metabolism.

This study generated valuable data toward understanding the effects of elevated temperature on organic acid metabolism. Heat-wave simulations in Shiraz berries resulted in temporary increases in malic acid when applied during early development, and lasting decreases in malic acid when applied during véraison and ripening. Tartaric acid levels were largely unaffected, but did also show a temporary increase with early-development treatments, which is thus likely due to an advancement of development in the heat-exposed berries. The loss of malic acid in véraison and ripening berries exposed to elevated temperatures was likely due to increased respiration through the up-regulation of mitochondrial TCA cycle genes, resulting in elevated levels of numerous amino acids; some of which were stress-related (e.g. proline and GABA). Based on altered transcript levels of tonoplast transporters, the susceptibility of malic acid to degradation was likely due to increased efflux of malic acid from the vacuole, however this needs to be tested further. Therefore, potential strategies for retaining malic acid in the berry may be to target the release of malic acid from the vacuole, although an exact mechanism for this is not immediately obvious. More needs to be known about the activities of these tonoplast transporters and their regulation.

Another strategy for altering malic acid levels in the berry during ripening may be to increase nitrogen availability in the soil, as the loss of malic acid during heat stress may occur, in part, to support amino acid biosynthesis. Thus, if amino acid levels are already high due to increased nitrogen incorporation during pre-véraison and early véraison, there may be less of a need for malic acid

metabolism during ripening to support amino acid biosynthesis. This hypothesis also needs testing, however previous studies have noted that increased vine nitrogen status can lead to increased malic acid concentrations (Keller *et al.*, 1998; Hilbert *et al.*, 2003). It has been suggested that the increased nitrogen leads to bulkier canopies, which reduces berry exposure to light and heat and could thus also reduce malic acid losses during ripening (Reynard *et al.*, 2011). Nitrogen itself can also increase the pH of the cell and thus activate malic acid synthesis as part of the cellular pH stat, which could also lead to higher concentrations of malic acid in the cytosol. A preliminary study using a newly developed technique for analysing cellular pH in vivo, demonstrated a temporary cytosolic acidification at véraison that may be caused by malic acid release from the vacuole. As yet the effect of the application of nitrogen fertilisers under controlled conditions or within single vineyards, with a focus on resultant amino acid and malic acid accumulation and metabolism, and cellular pH, remains unexplored. Nevertheless, improving grapevine nitrogen status, through exogenous application or enhancing the nitrogen use efficiency of cultivated grapevines, could be an important way forward and warrants additional research.

Aim 2: To examine the variability of responses to warming temperatures in selected cultivars and to develop predictive tools for acid management strategies to mitigate the problems associated with low acid levels in musts.

To study the effect of temperature on other *V. vinifera* cultivars differing in organic acid levels, numerous vines were screened for differing berry malic and tartaric acid levels. As the lower levels of malic acid seen at harvest are typically associated with exaggerated degradation by heat treatments during ripening, cultivars demonstrating different patterns of malic acid degradation were sought. Four cultivars that showed differing malic acid levels at harvest were used for closer developmental screens to determine how the patterns of organic acid accumulation and loss differed throughout development. These four cultivars were Palomino (a low-malic-acid, white cultivar), Chenin Blanc (a high-malic-acid, white cultivar), Cabernet Sauvignon (a low-malic-acid, red cultivar) and Tinta Cao (a high-malic-acid, red cultivar), as identified by Kliewer *et al.* (1967). Of these varieties, Tinta Cao berries clearly demonstrated lower rates of malic acid loss during ripening compared to the others. Based on transcriptional profiles, this could have been due to a higher level of sequestration of malic acid in the berry cell vacuole. This cultivar also demonstrated generally low levels of berry amino acids, suggesting that the sequestration of malic acid likely limited the biosynthesis of amino acids.

Tinta Cao, along with numerous other red cultivars, was used for cabinet-based, elevated- temperature treatments with excised canes. All cultivars demonstrated malic acid loss upon heating, as typically seen in Shiraz. However, this difference was only significant for Sangiovese and Shiraz, and not for Tinta Cao, Petit Verdot, Barbera, nor Cabernet Sauvignon. Furthermore, Tinta Cao berries still retained a higher level of malic acid than the other cultivars after heating, due to the high initial levels before the treatment. In the field, the large canopy of Tinta Cao may protect the berries from excessive light exposure and high temperatures, thus potentially further reducing the impact on malic acid levels. However, this remains to be tested at the whole-vine level. The large canopy of this cultivar was not likely to be due to enhanced nitrogen status compared to the other cultivars, as they were all sourced from the same vineyard, and Tinta Cao actually showed lower levels of amino acids in general, compared to the other cultivars.

Tinta Cao may serve as a useful cultivar in the quest for grapevines that retain high levels of malic acid during ripening and in response to elevated temperatures. Currently Tinta Cao is used in a very limited capacity for winemaking within Australia, and typically only for blending due to the high acid levels. However it may also serve as useful germplasm for yielding fruit with desirable acid compositional profiles under the higher temperatures predicted to occur in the viticultural regions of Australia in future years.

Aim 3: Characterise key steps in TA synthesis to identify potential control points that may be targeted for increased berry organic acid levels.

Enzymatic characterisation of a putative 2-keto-L-gulonate reductase as identified by Burbidge (2011) was carried out, and the product of the reaction with the expected substrate, 2-keto-L- gulonate yielded the expected product, L-idonate, according to GC/MS analysis. However, upon testing this enzyme with some additional substrates, it was clear that the enzyme had a preference for glyoxylate and hydroxypyruvate based on maximum reaction rates and affinities. Therefore the enzyme may typically function as a glyoxylate reductase, with a potential function as a 2-KGR when exposed to 2-KGA substrate. As the L-idonate dehydrogenase, which carries out the subsequent reaction of L-idonate conversion to 5-keto D-gluconate, was also shown to have diverged from an enzyme family with a completely different role in plant metabolism (Yong reference), and another step of the pathway is thought to be carried out by a transketolase of the pentose phosphate pathway or Calvin cycle, it may be that the biosynthetic pathway generally relies on alternative activities of enzymes from other metabolic pathways within the grape cell. This may not be surprising, considering that *V. vinifera* is one of the few species that accumulate tartaric acid and may rely on relatively recent evolutionary divergence of existing enzymes.

Unfortunately, two candidate genes for transketolase from grapevine could not successfully be shown to carry out transketolase activity, potentially due to inactivation during protein expression and purification in the bacterial expression system. Alternative expression systems will need to be tested in the future.

To further explore the roles of the three L-idonate dehydrogenase genes and the 2-keto-L-gulonate reductase candidate in tartaric acid biosynthesis, transgenic microvines were generated with the aim of overexpressing or knocking down the expression levels of these genes. Thus far, genotyping has confirmed the successful generation of two *VvLidh1* overexpressor lines, five *VvLidh2* overexpressor lines, seven *VvLidh3* overexpressor lines, potentially eighteen *VvLidh1* RNAi lines, potentially six *VvLidh1&2* double RNAi lines and six *Vv2kgr* RNAi lines. In addition, eight lines overexpressing a galacturonate reductase gene, involved in the synthesis of the tartaric acid precursor, ascorbate, and four lines overexpressing a transcription factor "Stop1" homolog that has been shown to influence the expression of organic acid transporters in *Arabidopsis thaliana* (Sawaki et al. 2009), were generated. Metabolic characterisation of these lines, which are at the T1 generation, is underway but not complete, to determine whether alterations in the expression of these genes result in altered accumulation of tartaric acid in the leaves or berries. Leaves may be a useful model for further investigating the effects on tartaric acid biosynthesis in these plants, as they were shown to accumulate equivalent, or even higher levels of tartaric acid when compared to the berry.

Meanwhile, a wild grapevine species *Ampelopsis aconitifolia*, which naturally lacks tartaric acid and L-idonate dehydrogenase gene transcript, was further explored with respect to the accumulation of other metabolites that might provide insights into the role of tartaric acid accumulation in the berry, and what other metabolites accumulate when tartaric acid cannot. According to previous study, these berries demonstrated extremely high levels of the tartaric acid precursor, ascorbate (DeBolt et al. 2006). In the present study a large accumulation of quinate was also seen in these fruit. A link between tartaric acid biosynthesis and quinate metabolism could arise through altered activities of the pentose phosphate pathway, glycolysis or shikimate pathway. Results from this experiment suggest that in the absence of tartaric acid, high accumulation of quinic acid (an astringent) may occur due to alterations in flux through carbon metabolism. Increasing tartaric acid accumulation may therefore draw on metabolites that would otherwise be used for other avenues of carbon metabolism and otherwise exhibit other sensory properties in the fruit. The only way forward with this is to examine the direct effects of manipulation of genes from the tartaric acid biosynthesis pathway, with respect to other metabolites of the fruit. It would be interesting to see whether *A. aconitifolia* expresses the gene candidates for other steps of the tartaric acid biosynthesis pathway, for example the 2-keto-L-gulonate reductase candidate.

Aim 4: To develop models obtained from whole-plant studies that describe the metabolism of tartaric and malic acids during grape berry development.

The transcript and metabolite data collected from this project were assembled into a number of models demonstrating malic acid metabolic pathways in different cultivars and in response to elevated temperature treatments. The most important finding from these models was a link between malic acid metabolism and amino acid metabolism, thus potentially explaining previous studies that have found a link between enhanced nitrogen supply and malic acid levels. This is the most relevant finding of this study in terms of immediate practical application, although the mechanism warrants further investigation.

A model was also generated to compare the metabolite profiles of tartrate-lacking *A. aconitifolia* berries with tartrate-accumulating *V. vinifera* berries, demonstrating a switch from tartrate accumulation to quinate accumulation in *A. aconitifolia* fruit that may be caused by the lack of the tartaric acid biosynthesis enzyme, L-idonate dehydrogenase. As yet it is unknown whether other candidate genes of the tartrate biosynthesis pathway are also lacking in this species. It also demonstrated a lack of malic acid, which seemed to be replaced by succinic acid and citric acid, implicating an interruption of the TCA cycle in this species. Again, it seems that the TCA cycle and anaplerotic use of malic acid for amino acid biosynthesis could be key to explaining malic acid levels in grape berries.

Aim 5: To provide to the Australian grape and wine industry with knowledge to permit the future selection of germplasm with the capability of yielding fruit with the desired acid compositional profiles when grown under the higher temperatures predicted to occur in the viticultural regions of Australia in future years.

Based on the information presented here, vine nitrogen status may play an important role in the regulation of malic acid metabolism during berry development and ripening between cultivars, and may even influence how the acid is affected by elevated temperatures. In order to confirm this, further testing is required with field-grown or potted vines of various cultivars, including Tinta Cao, to look more closely at nitrogen supply and the effect that this has on the loss of malic acid during elevated temperature treatments. As it is, Tinta Cao seems to be a useful cultivar for the Australian grape and wine industry to consider developing and adopting under rising seasonal temperatures, pending sensory analyses. As it is, a limited number of winemakers already use the cultivar in a blending capacity for achieving improved acidity (e.g. the d'Arenberg "Sticks and Stones" Tempranillo/Grenache/Tinta Cao/Souzao). These late-ripening vines show good vigour, large canopies,

large leaves and high fruit yield, and require minimal water and nutrient inputs (Dimasi, pers comm.).

Overall, the project achieved most objectives. The main shortfall was the limited analysis of the transgenic microvines, due to the time it took to generate the plant lines. The plants are currently being maintained in The Plant Accelerator, for molecular and metabolic analysis of leaf and berry tissues. In addition, progeny from a high- and low-acid cultivar cross that were established from canes in pots at the beginning of the project could not be used for fruit acid analyses, due to limited fruit crops over the course of the project. This was due to poor establishment of the canes as they had been in storage for an extended period, and the light conditions may not have been optimal in the shade-house used for their propagation. In place of this, a separate study was undertaken by a student, Emily Higginson, with a population from CSIRO, and additionally, cultivars from the field were selected for analysis based on widely varying organic acid levels at harvest, as measured by Kliewer *et al.* (1967) and in our own hands during a pilot trial. The latter approach enabled the thorough metabolic characterisation of Tinta Cao berries that demonstrated enhanced malic acid levels compared to other cultivars, over multiple seasons.

To summarise the findings in relation to the two main aims presented at the beginning of this section:

Important regulatory junctions of malic acid metabolism that may be targeted for reducing acid losses during hot periods of the season include *VvPepc2*, *VvNadme1*, *VvAlmt9* and *VvTdt* genes. However, according to comparisons of metabolite data between various *V. vinifera* cultivars, and in response to elevated temperature, altered nitrogen metabolism could also impact on malic acid levels in grape berries, and should be explored further as a potential regulator of berry acidity.

A new gene involved in the largely uncharacterised tartaric acid biosynthesis pathway could be fulfilled by a 2-keto-L-gulonic acid reductase candidate gene. This gene encodes an enzyme that is essentially a glyoxylate reductase enzyme with the potential to act as a 2-keto-L-gulonic acid reductase in the presence of the substrate. The generation of transgenic microvines that target this gene candidate and the previously characterised L-idonate dehydrogenase 1 gene, as well as two other isoforms of L-idonate dehydrogenase, will help us to determine whether these steps are rate- limiting for tartaric acid biosynthesis in grapevine, as ongoing work that stems from this project.

Post-crush additions of tartaric aid can reach as high as 8 g/L in hot seasons, costing up to $50 per 1000 L of juice. As recent growth in the Australian wine industry has led to the establishment of vineyards within hotter regions of Australia, overheads such as tartaric acid additions put the industry at

risk. More cost-effective winemaking strategies can contribute to sustained winemaking in such threatened areas. The enhancement of tartaric acid or malic acid concentrations in Shiraz grape berries for example, by 1.6 mg per gram (assuming ~0.8mL juice per gram), has the potential to reduce expenditure on tartaric acid applications by around 25%; that is, up to $4 million across the South Australian Riverland alone. The berries analysed in this study typically contained tartaric acid concentrations between 3-11 mg per gram, and malic acid concentrations between 0.5-6 mg per gram. Therefore, the enhancement of juice acidity at harvest may be achievable by crossing currently used cultivars, or manipulating metabolic pathways within existing cultivars by nutritional additions, to maintain higher concentrations of organic acids at harvest. The findings from this study identified gene targets that could be used to enhance organic acid concentration within existing cultivars, or that have potential in breeding strategies. However, further work is required to determine the effect of modulating these genes in grapevine, with respect to metabolic profiles of the fruit.

8. Recommendations

This project delivered three major outcomes in the form of knowledge advancement for the industry and research field.

First, the most practical and short-term possibility for manipulating malic acid levels is likely through altering nitrogen supply to the vines. Future research is required to determine whether altering the nitrogen supply at specific times during vine development will provide a repeatable improvement of malic acid levels under higher temperatures, and whether this is due to altered cytosolic pH or amino acid metabolism, or some other mechanism. Improving grapevine nitrogen status through exogenous application or enhancing the nitrogen use efficiency of cultivated grapevines, could be an important way forward and warrants a thorough and timely investigation to elucidate the mechanism by which nitrogen status affects malic acid, the optimum timing and concentration of practical applications, and a cost-benefit analysis.

Secondly, for a more specific manipulation of malic acid levels in response to elevated temperatures, genes encoding proteins involved in malic acid compartmentalisation and re-synthesis are likely candidates, namely a vacuolar aluminium-activated malate transported that is localised to the vacuolar membrane (*VvAlmt9*), a tonoplast dicarboxylate transporter (*VvTdt*) and a phosphoenolpyruvate carboxylase (*VvPepc2*).

Thirdly, after a metabolic analysis of four cultivars differing in malic acid accumulation and degradation patterns throughout berry development and ripening, and of numerous cultivars in response to elevated temperatures during véraison and ripening, Tinta Cao is recommended as a superior cultivar in terms of malic acid retention during berry ripening.

In addition, the academic implications of this work include a new method for the analysis of berry cell pH, which may have an important role to play in the physiology and biochemistry of véraison and berry ripening overall; the development of new transgenic lines targeting key enzymes of organic acid metabolism for biochemical and molecular analysis; and the recognition of the importance of amino acid metabolism in the response to elevated temperature stress in fruit. In addition, it is suggested that the biosynthesis pathway for tartaric acid may "borrow" enzymes from other metabolic pathways, including sorbitol dehydrogenases, glyoxylate reductases and transketolases, and therefore its manipulation may impact on other important pathways in the cell, including photosynthesis, the pentose phosphate pathway and shikimate pathway. To confirm this, transgenic plants generated within this project need to be thoroughly examined at the metabolite level before these genes can be considered as useful targets for potential breeding strategies. Future research with these transgenic microvines will also help to address the question of "how and why does tartaric acid accumulate in the grape berry, and how can it be regulated?"

9. Appendix 1: Communication

The findings of this study were presented in the form of oral presentations, scientific posters, and peer-reviewed publication, as listed below.

2011

Oral presentation by Crystal Sweetman at the Crush 2011 conference, Adelaide, Australia 28-30 September

2012

Facility visit and oral presentation by Christopher Ford at the James Hutton Institute, Dundee, Scotland, July 2013

Four scientific poster presentations at the ComBio 2012 conference, Adelaide, Australia, 23-27 September

2013

Oral presentations by Christopher Ford and Crystal Sweetman at the 9th International Symposium on Grapevine Physiology & Biotechnology Conference, La Serena, Chile, 21-26 April

Two scientific poster presentations at the 9th International Symposium on Grapevine Physiology & Biotechnology Conference, La Serena, Chile, 21-26 April

Facility visit and oral presentation by Crystal Sweetman at The Institute of Agricultural Research (INIA) La Platina, Santiago, Chile, 15 April

Facility visit and oral presentation by Crystal Sweetman at The University of Chile, Santiago, Chile, 16 April

Facility visit and research activities by Crystal Sweetman at the James Hutton Institute, Dundee, Scotland, 3 May–1 June

Facility visit and oral presentation by Crystal Sweetman at The University of La Rioja, Logroño, Spain, 3 June

Facility visit and oral presentation by Crystal Sweetman at the National Institute for Agricultural Research (INRA) Science Institute of Vine and Wine (ISVV), Bordeaux, France, 10 June

Facility visit and oral presentation by Crystal Sweetman at the National Institute for Agricultural Research (INRA), Colmar, France, 12-13 June

Oral presentation by Christopher Ford at the ComBio 2013 conference, Perth, Australia, 29 September–3 October

Scientific poster presentation by Crystal Sweetman at the 16th Australian Wine Industry Technical Conference (AWITC), Sydney, Australia, 14-17 July

Oral presentation by Crystal Sweetman at CSIRO Plant Industry, Adelaide, Australia on 5 November

2014

Publication of peer-reviewed manuscript in the Journal of Experimental Botany, 1 September (Sweetman *et al.*, 2014)

10. Appendix 2: Intellectual Property

No intellectual property arose from the research.

11. Appendix 3: References

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12. Appendix 4: Staff

Staff engaged on this project were:

Assoc. Prof. Christopher Ford – Project Supervisor

Dr Crystal Sweetman – Chief Investigator

Dr Jake Dunlevy – Research Assistant

Assoc. Prof. Colin Jenkins – Research Assistant

Ms Denise Ong – Research Assistant

Ms Karen Francis – Research Assistant

Ms Mai Nguyen – Research Technician

Mr Martin Rossa – Research Technician

Assoc. Prof. Kathleen Soole – Collaborator

Assoc. Prof. Victor Sadras – Collaborator

Dr Robert Hancock – Collaborator

13. Appendix 5: Additional Material

Table A5.1: Primer sets used for the generation of various expression constructs for the transformation of microvines. OEX = overexpression, RL = Restriction-ligation, TOPO = F primer contains CACC sequence for cloning with D-TOPO vector. ATT = with Att primer sequence (given separately)

Target	Accession	Application	F primer sequence (5' à 3')	R primer sequence (5' à 3')	Amplicon (bp)
VvPepc2	XM_002280806	OEX, RL	GAGAGTCGACATGAC GGACACCACAGATG	TATACCCGGGGTTGGAG TGCAGCAGTTTC	3206
VvStop1	XM_002270160	OEX, RL	GAGAGTCGACATGGA TTCTCAAAGATAGG	TATACCCGGGCTACTCA AGATCATTAGAAC	1604
VvGalUR	NM_001281196	OEX, RL	GAGA**GTCGAC**ATGGC AAAGGCACCTCAAG	TA**TCTAGA**TCAAATCTCA TCGTCAAGCTG	967
VvLidh1	XM_002269900	OEX, TOPO	CACCAGAGATGGGGA AAGGAGGCA	ACGCTTAGAAACATAGC CTGA	1135
VvLidh2	XM_002269859	OEX, TOPO	CACCGCTGCTGTGTCT TCCGTCAC	CCATCTAGACTGGTAAG ACTGTT	1265
VvLidh3	XM_002267626	OEX, TOPO	CACCGCTGAGACCGA GGGACAAGAG	CAGCAAGCCGAAAGTCA CTCCT	1209
VvNadme2	XM_002266661	RNAi, ATT	GGTTACATGACAGAA ATGAGACAATG	CCAATGCCCTGAACTCC	275
VvLidh1	XM_002269900	RNAi, ATT	TCAAGATTCAACCCTA CATTC	CATTTCTCTGCATAGATT GTATTG	275
VvLidh1and2	XM_002269900 XM_002269859	RNAi, ATT	TTCAACGAATATTCAG GATC	CCACGAGCACTGGTTTC	363
Vv2Kgr	XM_003632812	RNAi, ATT	AGCCGAGGGATTTAG CTGTC	CCGATGTTGATGACCAC ACC	224
Att Primer attachments			GGGGACAAGTTTGTA CAAAAAAGCAGGCTC	GGGGACCACTTTGTACA AGAAAGCTGGGTC	

Figures A5.1-5.35: Metabolite data from output 3.

A set of 35 graphs showing metabolite data from experiments described on pp 58-65. These data have been withheld from the Final Report pending the preparation of a manuscript. Further information is obtainable from the Project CI.

Appendix 5.1: Attempt to confirm activity of PPDK in grape berry tissue

During malic acid breakdown around véraison, various pathways of malate metabolism have been suggested (Sweetman *et al.*, 2009). Transcript analysis has shown the potential involvement of a number of genes and activities of several of the encoded enzymes has been demonstrated in grape extracts. An important enzyme potentially involved in the malate pathway is pyruvate, Pi dikinase (PPDK). Transcript levels of this gene were again shown to be up-regulated in véraison berries for most cultivars in the present work (Outputs 2 and 3). However, while the enzyme expression was shown at the transcript level, no activity of this enzyme has been demonstrated in grape extracts. This enzyme, characterised extensively in so-called C_4 plants, such as maize, has been difficult to measure due to its complex post-translational regulation systems, involving phosphorylation and thiol-disulphide oxidation, as well as cold sensitivity. Activities can be measured in C_4 plants, using a spectrophotometric method, by coupling the product PEP to NADH oxidation via PEP carboxylase and malate dehydrogenase (Ashton *et al.*, 1990). Its occurrence at low levels has been occasionally demonstrated also in C_3 plants, generally by Western blotting or sometimes by a very sensitive radiolabelling assay. In C_3 plants the enzyme presumably has an anaplerotic role. We attempted to measure this activity in grape extracts using a spectrophotometric method. PEP carboxylase was part purified from maize to use for assays, and low levels of PPDK activity could be shown from maize. However, no PPDK activity could be detected in grape extracts, which were concentrated by PEG precipitation as usual for measuring other enzyme activities. Presumably the enzyme activity in grapes is very low and undetectable by this method, or sensitive to the preparation conditions, and/or sensitive to complex regulation as in other plants. Other studies have also reported a lack of PPDK activity from grape berry enzyme preparations (Famiani *et al.*, 2014).